University of Alberta

Characterization of chemokine CCL19 in the duck (*Anas platyrhynchos*)

by

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To my parents

Abstract

A sequence with similarity to mammalian chemokine CCL19 was identified in an expressed sequence tag project to identify duck genes relevant to immunity. Mammalian CCL19 recruits antigen presenting cells and lymphocytes to the secondary lymphoid organs. Northern hybridization indicated significant expression of the transcript in spleen and lung. Southern blot analysis showed two hybridizing bands, suggesting CCL19 most likely exists in two copies in the duck genome. The sequence encoding the mature protein was directionally cloned into the pET29b vector with a 6X Histidine-tag at the carboxyl terminus. Recombinant duck CCL19 made in a bacterial expression system was purified by affinity column chromatography and tested for biological activity *in vitro*. A gradient of CCL19 attracted duck peripheral blood leukocytes in a transwell migration assay.

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List of Abbreviations

Ag/Ags	Antigen/Antigens
APC	Antigen presenting cell
BLR	Burkitt's lymphoma receptor
CCL	Chemokine ligand
CCR	Chemokine receptor
CTL	Cytotoxic T cell
DC	Dendritic cell
DHBV	Duck hepatitis B virus
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus encoded nuclear activator
EC	Extracellular
ECL	Extracellular loop
ELC	Epstein-Barr virus induced ligand chemokine
ELR	Glu-Leu-Arg
EST	Expressed sequence tag
FBS	Fetal bovine serum
GAG	Glycosaminoglycans
GDP	Guanine di-phosphate
GEF	Guanine nucleotide exchanging factor
GPCR	G protein coupled receptor
GTP	Guanine triphosphate
HBV	Hepatitis B virus

HBsAg	Hepatitis B surface antigens
HCV	Hepatitis C virus
HEV	High endothelial venule
HPV	Human papilloma virus
HSV	Herpes simplex virus
HHSV	Human Herpes simplex virus
IC	Intracellular
ICL	Intracellular loop
IL	Interleukin
LN	Lymph node
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MIP-3β	Macrophage inflammatory protein
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBL	Peripheral blood leukocyte
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RT	Reverse transcriptase
TAA	Tumour associated antigen
TM	Transmembrane
TNF	Tumour necrosis factor

1. INTRODUCTION

1.1 Chemokines

The generation of protective immunity against pathogens and the effective maintenance of immune surveillance is manifested by the orchestrated movement of leukocytes within distinct microenvironments (Nagira et al. 1997; Dieu et al. 1998; Gunn et al. 1998; Forster et al. 1999; Sallusto and Lanzavecchia 2000). The key players in guiding the leukocytes through the tissues and peripheral lymphoid organs are small, secreted polypeptides known as chemokines (Ebert et al. 2005; Limatola et al. 2005). Chemokines are a family of specialized cytokines with a molecular weight of 8-12 kDa, that primarily mediate leukocyte trafficking under basal and inflammatory conditions (reviewed by Fernandez and Lolis 2002; Campbell et al. 2003; Gunn 2003; Laing and Secombes 2004; Moser et al. 2004). All the chemokines are structurally related and characterized by the presence of highly conserved cysteine domains. The entire chemokine family is classified into CC, CXC, CX_3C and C subtypes based on the configurations of the cysteine residues at the amino terminus [Table 1] (Rossi and Zlotnik 2000; Fernandez and Lolis 2002).

Chemokines possess diverse biological functions (Campbell et al. 2001; Reiss et al. 2001). Based on their function and expression, chemokines are subdivided into two categories – constitutive and inflammatory or inducible (Moser and Loetscher 2001; Kunkel and Butcher 2002). The former are constitutively expressed in the secondary lymphoid tissues and serve a

housekeeping function. They facilitate lymphocyte homing into the lymphoid organs to undergo activation and also maintain homeostasis ie. the number of lymphocytes circulating between the tissues and the peripheral lymphoid organs (Campbell et al. 1999; Peled et al. 1999; Zabel et al. 1999; Kunkel and Butcher 2002; Uehara et al. 2002). The inflammatory or inducible chemokines serve as the principal molecular cues in navigating specific subsets of leukocytes between the tissues and secondary lymphoid organs in inflammatory immune responses. Their expression is strongly upregulated in the peripheral tissues in response to inflammatory stimuli like LPS, IL-1 and TNF-α (Luster and Ravetch 1987; Sherry et al. 1988; Leonard and Yoshimura 1990; Schall et al. 1990; Bazzoni et al. 1991; Jose et al. 1994).

The ability of leukocytes to respond to chemokines by sensing the chemotactic gradients is conferred by chemokine receptors. They are members of the 7 transmembrane spanning G protein coupled receptors (Rollins 1997; Rossi and Zlotnik 2000). The differential expression of chemokine receptors on any given leukocyte type directs their entry from the peripheral tissues into the lymph node (Sozzani et al. 1998; Ohl et al. 2004; Bromley et al. 2005; Debes et al. 2005). Certain chemokines belonging to the CXC group are also involved in angiogenesis/angiostasis (Koch et al. 1992; Strieter et al. 1995; Karl et al. 2005) as well as tumour growth and metastasis (Arenberg et al. 1997; Luan et al. 1997). Thus, the regulated expression of chemokines and chemokine receptors aid leukocyte development, maintain leukocyte homeostasis and control leukocyte trafficking in order to sustain immunity.

Chemokine nomenclature	
Chemokine	Receptor
CC chemokines	
CCL1	CCR8
CCL2	CCR2
CCL3	CCR1,CCR5
CCL4	CCR5
CCL5	CCR1,CCR3,CCR5
CCL7	CCR1,CCR2,CCR3
CCL8	CCR3
CCL11	CCR3
CCL13	CCR2,CCR3
CCL14	CCR1
CCL15	CCR1,CCR3
CCL16	CCR1
CCL17	CCR4
CCL18	Unknown
CCL19	CCR7
CCL20	CCR6
CCL21	CCR7
CCL22	CCR4
CCL23	CCR1
CCL24	CCR3
CCL25	CCR9
CCL26	CCR3
CCL27	CCR10
CXC chemokines	
CXCL1	CXCR2,CXCR1
CXCL2	CXCR2
CXCL3	CXCR2
CXCL4	Unknown
CXCL5	CXCR2
CXCL6	CXCR1,CXCR2
CXCL7	CXCR2
CXCL8	CXCR1,CXCR2
CXCL9	CXCR3
CXCL10	CXCR3
CXCL11	CXCR3
CXCL12	CXCR4
CXCL13	CXCR5
CXCL14	Unknown
C chemokine	
XCL1	XCR1
XCL2	XCR1
CX3C chemokine	
CX3CL1	CX3CR1

Table 1. Systematic nomenclature of chemokines

1.2 Brief evolutionary history of chemokines

The origin and evolutionary history of chemokines has been traced back to about 650 million years ago [mya] in early vertebrates (DeVries et al. 2006). The chemokine system has been completely characterized and extensively described only in mice and humans (Muller et al. 1995; Imai et al. 1996; Nagira et al. 1997; Rossi et al. 1997; Nakano et al. 1998; Ngo et al. 1998; Vassileva et al. 1999; Stein et al. 2000; Nakano and Gunn 2001). With the advent of bioinformatics and using an expressed sequence tag [EST] based approach, about 42 chemokines and 18 chemokine receptors have been identified in humans (Rollins 1997; Rossi and Zlotnik 2000; Laing and Secombes 2004; DeVries et al. 2006). Apart from mice and humans, about 25 different C, CC, CXC and CX_3C chemokines have been identified in the rhesus monkey (Basu et al. 2002). Overall, there is an indication of evolutionary conservation among the chemokine subfamilies across different mammalian vertebrate species.

However, the chemokines are not well characterized in non mammalian vertebrates. There have been a few reports in fishes suggesting that chemokines originated early in the evolution of vertebrates (Lally et al. 2003; Laing and Secombes 2004; Mackenzie et al. 2004; Baoprasertkul et al. 2005; Goostrey et al. 2005; Inoue et al. 2005; Peatman et al. 2005; Peatman et al. 2005). EST databases in channel catfish and blue catfish have revealed many CC and CXC chemokines as multiple ESTs (He et al. 2004). A number of putative chemokines have been identified and reported from the draft genome of zebrafish (DeVries et al. 2006). Although there are more chemokines and chemokine receptors than found in mice

and humans, the data are fragmentary. Several chemokines such as CCL1, CCL3, CCL6, CCL7, CCL8, CX_3CL1 reported in humans and mice are lacking in zebrafish. It is interesting to note that many of these chemokines are involved in T cell differentiation and the initiation of T helper (Th_2) type of immune responses (DeVries et al. 2006). A similar analysis on the pufferfish genome indicated fewer chemokines and chemokine receptors (Aparicio et al. 2002).

The birds and mammals shared a common ancestor about 310 mya (Kumar and Hedges 1998). The chicken draft genome and the availability of the chicken EST database have facilitated the systematic identification, characterization and annotation of chemokine and chemokine receptor genes in the chicken genome (Wang et al. 2005; DeVries et al. 2006). About 23 chemokines encompassing the C, CC, CXC and CX_3C subfamilies and 14 chemokine receptors have been reported in the chicken (Hughes and Bumstead 2000; Sick et al. 2000; Hughes et al. 2001; Smith et al. 2004; Kaiser et al. 2005; Wang et al. 2005). The phylogenetic analyses suggested that most of the chicken chemokine and chemokine receptor genes shared a common ancestry with the mouse and human chemokine and chemokine receptor families. Also, there were significantly more gene duplications among the CC, CXC, CCR and CXCR subfamilies in mammals than in aves after their divergence (Kaiser et al. 1999; Hillier et al. 2004; Wang et al. 2005). Among the other birds, only three different chemokines namely CCL4, CCL5 and RANTES have been identified in ducks (Sreekumar et al. 2005).

1.3 Molecular organization of the chemokine system - classification and nomenclature based on structure

Chemokines are structurally related proteins with most members containing four invariant cysteines at the amino terminus (Rollins 1997; Laing and Secombes 2004). They are classified into two large subgroups CC, CXC and two small subgroups C and CX_3C based on the number and arrangement of the cysteine residues [Table 1] (Baysal and Atilgan 2001; Fernandez and Lolis 2002). The members of the CXC and CX_3C subgroups contain one or three amino acids respectively in between the cysteine residues. Among the CC chemokines, the cysteine residues are located adjacent to each other. The C chemokine lymphotactin, is exceptional since it contains only a single cysteine residue in the N terminal domain. Another exception is fractalkine, an integral membrane protein which has three amino acids intervening between the first two cysteines (Rollins 1997; Rossi and Zlotnik 2000). The CXC chemokines are further subdivided into ELR and non-ELR subtypes. The ELR chemokines contain a three amino acid motif comprising of glutamate, leucine and arginine between the N terminus and the first cysteine, example - CXCL8 which recruits neutrophils (Baggiolini et al. 1994; Laing and Secombes 2004). The non-ELR CXC chemokines recruit different subsets of lymphocytes (Rollins 1997; Fernandez and Lolis 2002). Some of the genes encoding chemokines have been mapped and are found to cluster together at specific loci. In humans, the CC chemokines cluster at chromosome 17q11.2-12 and the CXC chemokine genes at 4q13 (Rollins 1997).

1.4 Structural biology of chemokines

NMR and X ray crystallography have enabled elucidation of the tertiary structure of certain chemokines. The mature chemokine domains are usually about 66-111 amino acids in length. Despite low sequence identities ranging from 20%-30% between the different chemokine subfamilies, the tertiary structure of all chemokines are remarkably similar [Fig1]. The tertiary structure is composed of three antiparallel β strands; β_1, β_2 and β_3 folded and packed by hydrophobic interactions against an amphipathic C terminal α helix (Ye et al. 1999; Baysal and Atilgan 2001; Ye et al. 2001; Handel et al. 2005). The region before the β_1 strand is divided into two segments, containing a disorganized N terminus and a comparatively well organized N - loop. These two segments are connected by a short peptide domain containing two to five amino acid residues terminated on each side by two of the four conserved cysteines. The section connecting the β_1 and β_2 strands is known as the 30s loop and has been implicated in receptor binding and/or activity. The loop connecting the β_2 and β_3 strands is called the 40s loop. (Clark-Lewis et al. 1994; Clark-Lewis et al. 1995; Baysal and Atilgan 2001; Rajarathnam et al. 2001). The unordered N terminal domain is usually variable in length and is critical for receptor activation (Ott et al. 2004 a; Ott et al. 2004 b). In the CXC chemokines, the ELR motif is located in this region (Clark-Lewis et al. 1994; Clark-Lewis et al. 1995). The C terminal α helix is highly basic in most chemokines and usually contains amino acid residues that bind to glycosaminoglycans [GAGs].

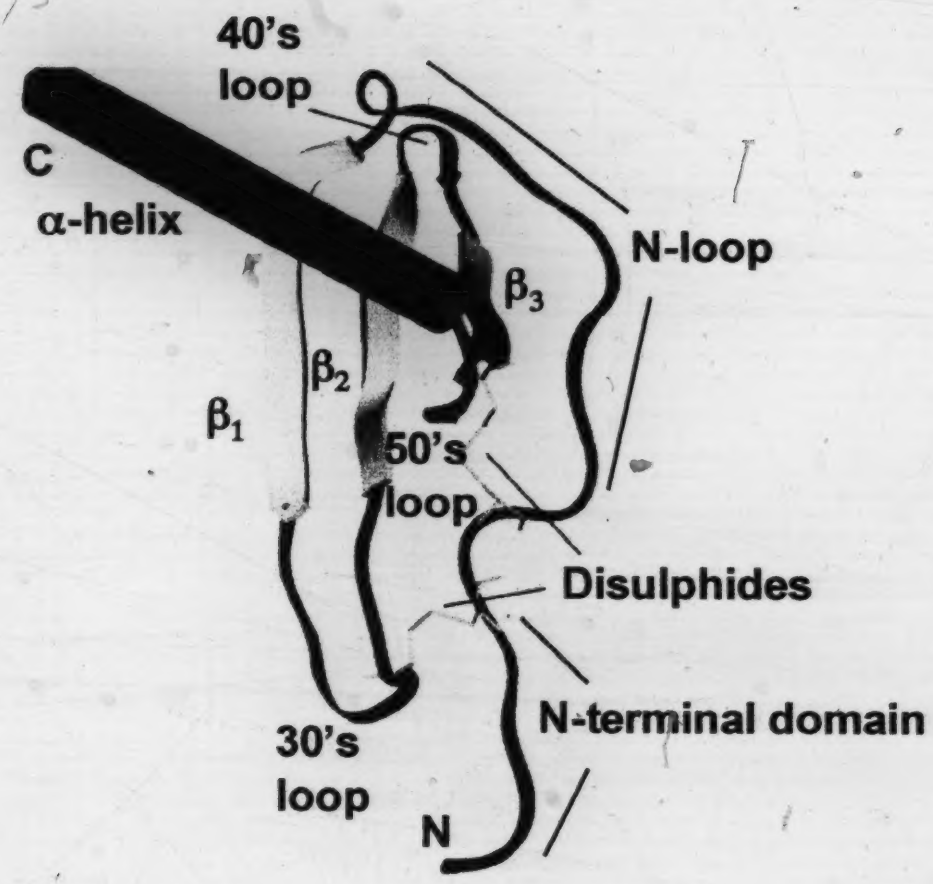

Fig. 1. Monomeric structure of the chemokine CXCL8. This is a ribbon diagram of CXC chemokine CXCL8 in which the different secondary structure elements are labeled accordingly (Adapted from Fernandez and Lolis 2002).

Apart from binding to their cognate G protein coupled chemokine receptors on migrating cells, it has been postulated that chemokines bind to different kinds of immobilized gycosaminoglycans [GAGs] selectively, as a mechanism for retention at the cell surface and presentation to circulating leukocytes (Kuschert et al. 1999; Middleton et al. 2002; Shriver et al. 2002). The glycosaminoglycans are a family of anionic polysaccharides located on the endothelial surface and the extracellular matrix (McCornack et al. 2004; Handel et al. 2005; Johnson et al. 2005). The importance of chemokine-GAG interactions is supported by experimental observations that chemokines bind to purified GAGs *in vitro* and *in vivo* (Middleton et al. 1997; Ali et al. 2000; Middleton et al. 2002). The majority of chemokines are highly basic and presumably bind to the negatively charged GAGs by nonspecific electrostatic interactions. However, studies in CCL4 and CCL5 suggest that the interaction between the chemokines and GAGs is specific (Proudfoot et al. 2003). Specificity has been observed in the relative affinity of some chemokines for heparin and other members of the GAG family (Hoogewerf et al. 1997; Kuschert et al. 1998). The interaction of chemokines with GAGs has been used as a novel therapeutic intervention. Heparin has anti-inflammatory disease ameliorating properties (Lever and Page 2002). Although the exact mode of heparin action is not well established, it is hypothesized that the interaction between pro-inflammatory cytokines and membrane associated GAGs may induce clinical immunosuppression. It is likely that the anti-inflammatory effects of heparin are mediated partly by interference with chemokines (Douglas et al. 1997; Douglas et al. 1997; Johnson et al. 2004).

The formation of disulphide bonds is of paramount importance in the stabilization of tertiary structures of chemokines as well as their biological activities (Clark-Lewis et al. 1994; Clark-Lewis et al. 1995; Nardese et al. 2001; Rajarathnam et al. 2001). Chemokines are quite stable in biological fluids, partly due to the disulphide bridges formed between Cysteines 1 and 3, and Cysteines 2 and 4. (Clore and Gronenborn 1995). Some members of the CC chemokines namely CCL14, CCL15 and CCL21 possess a third disulphide bond connecting the C terminal α helix to the β strand. However this does not appear to be critical for protein folding or function (Hedrick and Zlotnik 1997; Hromas et al. 1997; Tanabe et al. 1997; Hromas et al. 1999; Forssmann et al. 2001).

Three major types of quartenary structures have been reported in chemokines, monomers, dimers and tetramers. There are two distinct kinds of dimers, the compact CXCL8 common to several CXC chemokines made up of a six strand antiparallel β sheet (Clark-Lewis et al. 1994; Clark-Lewis et al. 1995; Baysal and Atilgan 2001; Swaminathan et al. 2003) and the extended CCL4 type found in many CC chemokines containing an antiparallel β sheet arrangement between the N terminal domains and N loops of the monomers (Clore and Gronenborn 1995; Baysal and Atilgan 2001). Recent experimental evidence suggest that GAGs can induce dimerization at low chemokine concentrations (Ali et al. 2001; Forssmann et al. 2001). In particular, GAG induced dimerization of CCL2 is critical for pro-inflammatory functions *in vivo* (Proudfoot et al. 2003).

1.5 Chemokine receptors

The migration of specific leukocyte subsets in response to inflammatory stimuli or under basal conditions is tightly regulated by the differential expression of chemokines and chemokine receptors (Kuziel et al. 1997; Sallusto and Lanzavecchia 2000; Uehara et al. 2002; Bromley et al. 2005; Randolph et al. 2005; Randolph et al. 2005). Similar to the classification system of chemokines, the chemokine receptors are classified into four main families, CR, CCR, CXCR and CX_3CR that interact with C, CC, CXC and CX_3C chemokines respectively (Murphy 1994; Murphy et al. 2000). The chemokine receptors are G protein coupled, seven transmembrane receptors that range between ~340-370 amino acids in length and are present on the membrane of target cells that enable them to respond to chemotactic cues. The N terminus is extracellular and the C terminus is intracellular. There are seven predicted membrane spanning domains, three extracellular loops [ECL] and three intracellular loops [ICL] (Rossi and Zlotnik 2000; Ott et al. 2004; Ott et al. 2004). Although similar in structure to many other seven transmembrane receptors, chemokine receptors possess certain unique structural features namely the amino acid motif DRYLAIV in the second intracellular loop domain (Murphy 1994; Rossi and Zlotnik 2000). The intracellular and hydrophobic domains of the chemokine receptors are highly conserved across species while the extracellular domains are the least conserved. In addition to the sequence motif DRYLAIV in the ICL2, the chemokine receptors have other common features like the presence of an acidic and relatively

short N terminal segment, a short basic ICL3 and a cysteine in each of the four extracellular domains (Mayer and Stone 2001; Limatola et al. 2005).

The chemokine receptor-ligand interactions are very promiscuous, with every chemokine receptor binding to a number of different chemokine ligands [Table1]. The chemokine receptors belong to the G protein coupled receptor family [GPCR] (Gao et al. 1993; Murphy 1994; Combadiere et al. 1995; Tiffany et al. 1997). The biochemical pathways underlying chemokine receptor-ligand interactions have not been characterized very well due to the lack of chemokine specific reagents.

Most of the knowledge about chemokine signaling has been acquired from the information available on GPCR signaling. The G proteins are plasma membrane associated signal transducers consisting of an α subunit which binds the guanine nucleotides GDP or GTP, and a covalently linked $\beta\gamma$ heterodimer subunit (Mellado et al. 2001 a; Mellado et al. 2001 b). The standard caveat for initiation of intracellular signaling through GPCRs requires activation of the G protein pathway upon chemokine ligand binding to the receptor. In an inactive or resting state, the GDP is bound to the α subunit. Upon ligand binding and receptor dimerization, the receptor is transformed into a guanine nucleotide exchange factor [GEF] which catalyzes the exchange of GDP for GTP on the α subunit. This causes the G protein to dissociate into α and $\beta\gamma$ subunits thereby activating downstream effectors, namely the JAK/STAT cascade. The conformational changes imposed by ligand binding followed by dissociation of the heterotrimeric G protein complex results in the activation of intracellular

signaling responses on distinct effector molecules. The α subunit has intrinsic GTPase activity which restores the GDP bound form of the α subunit and reassociates with the βγ subunits, thus completing the cycle (Mellado et al. 2001 a; Mellado et al. 2001 b; Soriano et al. 2003). Chemokine receptor-ligand interactions culminate in the migration of leukocytes towards the source of the chemokine due to redistribution of the cell cytoskeleton.

1.6 Mechanism of chemotaxis

The migration of leukocytes in response to chemotactic gradients is critical for leukocyte function and has implications in the initiation of inflammatory immune responses. Cell migration is a complex process that begins with the acquisition of polarity marked by morphological changes and a migratory phenotype. During this process, filamentous actin is segregated and concentrated in two specific distinct regions of the cell, the leading edge and the uropod. The uropod is a pseudopodia like projection which is important in cell adhesion and motility. In migrating cells, adhesion molecules like L-Selectin, ICAM, PSGL-1, Mac-1 and CD43 are concentrated in the uropod. This promotes binding and interactions between the neighbouring cells, thus enabling leukocyte recruitment and transendothelial migration (Sánchez-Madrid and del Pozo 1999). The leading edge concentrates various receptors such as those for integrin, chemokine receptors CCR2, CCR5 and CXCR4 in lymphocytes. The redistribution of chemokine receptors to the leading edge of the cell is initiated by many chemokines and few cytokines like IL-2 and IL-15. The presence of chemokine

receptors at the leading edge are not only important to establish polarity but also help the migrating cell to detect the chemotactic gradient and orient the cell towards the source of the chemoattractant (del Pozo et al. 1997; Sanchez-Madrid and del Pozo 1999). The molecules involved in signal transduction coupled to the activated chemokine receptors are also localized at the leading edge, implying their role in signaling the cytoskeleton directly in order to initiate cell migration (Parent et al. 1998).

1.7 Introduction to CCL19

The CC chemokines or β chemokines as they are commonly known, are characterized by the presence of two consecutive cysteines in the N terminus (Rollins 1997; Moser et al. 2004). Most CC chemokines are potent chemoattractants for monocytes. They are also known to attract lymphocytes, basophils and/or eosinophils with variable specificity and potency. Generally, the CC chemokines contain four cysteines, but there are certain exceptions. Some chemokines within the CC subfamily contain six cysteines. These include CCL1, CCL15, CCL21, CCL23 and CCL28 (Wang et al. 2000; Laing and Secombes 2004). The position of all the four cysteine residues among the various members of the CC chemokine family are highly conserved. The CC chemokines are 20%-30% identical at the amino acid level across different vertebrate species (Yoshida et al. 1997). Bioinformatics has paved way for the identification of several members of the chemokine family. One such chemokine identified in a large database of expressed sequence tags [ESTs] is the CC chemokine CCL19, also

known as ELC [Epstein-Barr virus induced ligand chemokine] or MIP-3 β [macrophage inflammatory protein- 3 β] (Rossi et al. 1997). By screening ESTs derived from the cDNA library of mouse thymus, a mouse CC chemokine was identified. This chemokine sequence was used as a query sequence to perform TBLASTN searches against the dbEST database. As a result, several human ESTs were discovered, that formed a contig. BLASTX analysis of the consensus sequence revealed that it encoded a unique human chemokine with a CC motif. This chemokine was designated as MIP-3 β then, but according to the nomenclature system currently in use, it is now known as CCL19 (Rossi et al. 1997).

CCL19, a potent leukocyte chemoattractant is a small secreted polypeptide with a molecular weight of approximately 10-12 kDa. The mammalian homologues of CCL19 have been well characterized. In humans, CCL19 is constitutively expressed in the lymphoid tissues as established by Northern blot analysis. The thymus, appendix and lymph nodes have been revealed as exceptionally rich sources of CCL19 in humans (Rossi et al. 1997; Yoshida et al. 1997). In mice, CCL19 is significantly expressed in the spleen, peripheral lymph nodes and fairly expressed in the mesenteric lymph nodes. In particular, mouse CCL19 was found to be distributed in distinct T zone dendritic cells [DCs] of spleen and lymph node as determined by *in situ* hybridization analysis (Ngo et al. 1998).

CCL19 is a dual chemokine exhibiting a homeostatic function as well as serving as a proinflammatory peptide. It regulates the trafficking of naïve and

some memory T lymphocytes, along with mature DCs into the T cell areas within the secondary lymphoid organs under resting and inflammatory conditions (Dieu et al. 1998; Ngo et al. 1998; Kunkel and Butcher 2002; Yoshida et al. 1998). The functional role of CCL19 is discussed in detail in section 1.13.

1.8 Identification of CCL19 and its cognate receptor CCR7

CCL19 was first reported in humans, named as EBI-1 ligand chemokine [ELC] (Yoshida et al. 1997). EBI-1, Epstein-Barr virus [EBV] induced gene was identified as an orphan, seven transmembrane spanning GPCR in strongly upregulated EBV-negative Burkitt lymphoma cell lines upon infection with EBV (Birkenbach et al. 1993). The same receptor was reported independently as Burkitt's lymphoma receptor 2 [BLR2] which was shown to be induced by EBV–encoded transactivator, EBNA-2 (Burgstahler et al. 1995). The gene encoding the receptor was later isolated from CD4 T cells that were infected with human herpes virus HHV6 and HHV7 (Hasegawa et al. 1994). It has been demonstrated that ELC specifically binds to EBI-1 with high affinity and induces chemotaxis and calcium mobilization in EBI-1 transfected cells (Yoshida et al. 1997). Thus, ELC or CCL19 was found to be the high affinity ligand for EBI-1, now termed as CCR7.

1.9 Structure of CCL19

As established by NMR and X-ray crystallography, all the CC chemokines have very similar three dimensional structures. A disordered N terminus that

precedes the conserved cysteines has been shown to be involved in stabilizing the active conformation of several CC chemokines. Following the conserved cysteines is the N-loop which terminates in one turn of a 3_{10} helix, a three stranded antiparallel β sheet and a C terminal α helix. The N terminus and the N-loop are tethered to the β sheet by the conserved disulphide bonds while the α helix lies in close proximity (Ott et al. 2004 a; Ott et al. 2004 b).

1.10 Mechanism of ligand binding and receptor interaction

Chemokines exert their biological effects through two separate sets of interactions namely interactions involved in high affinity binding and interactions critical for stabilizing the active conformation of the receptor. On the chemokine, the N-loop and loops connecting the antiparallel β strands are considered important for high affinity ligand binding. This has been demonstrated by the chemical synthesis of N-terminal analogues of CCL19. Successive truncations of the N-terminus of CCL19 resulted in decreased affinity of the receptor CCR7 for the ligand. The chemically synthesized N-terminal analogues of CCL19 were less potent in comparison to the wild type CCL19 in inducing chemotaxis of cell lines transfected with CCR7 (Ott et al. 2004 a). The N terminal regions are thought to interact with the extracellular domains of the receptor. The active conformation of the receptor is stabilized by low energy interactions between the N-terminal domain and the helix bundle of the receptor (Ott et al. 2004 b). However, the above mechanisms are not absolute for all chemokines and can vary in different members. For example, CCL19 contains residues involved in high affinity ligand

binding in the N terminus while the residues in the N-loop are implicated in receptor activation in CCL19 and CCL21. However, mutation of a lysine to alanine on CCR7 reduces CCL21 mediated receptor activation drastically by twenty two fold while CCL19 mediated activation is unimpaired. This indicates that there is at least one amino acid residue that distinguishes between interactions of CCL19 and CCL21 with CCR7 (Ott et al. 2004 a; Ott et al. 2004 b).

1.11 Vertebrate CCL19 and CCL21 – ligands of CCR7

A closely related vertebrate homologue of CCL19 is another CC chemokine CCL21 also known as secondary lymphoid-tissue chemokine [SLC] or Exodus 2 or Thymus-derived chemotactic agent 4 [TCA4]. The mammalian CCL21 homologues were first identified in an EST based approach and characterized (Hedrick and Zlotnik 1997; Hromas et al. 1997). The primary amino acid sequence of CCL21 is highly conserved among humans, mice and pigs. An important characteristic feature of CCL21 is the presence of an extremely basic, longer carboxy-terminal domain which is conserved in both mice and humans. Apart from the four cysteine residues found in the mature protein of all CC chemokines, the carboxyl terminus of CCL21 contains two additional cysteines. The disulphide bridge formed between these two cysteines result in the formation of a C terminal structure unique to CCL21. CCL19 lacks the additional highly basic C terminal domain found in CCL21 (Hedrick and Zlotnik 1997; Hromas et al. 1997; Hromas et al. 1999). CCL19 and CCL21 are structurally related chemokines and both bind to the receptor CCR7 which is expressed primarily on

mature DCs and naïve T cells (Campbell et al. 1998; Yoshida et al. 1998). The amino acid sequence of CCR7 was aligned with the amino acid sequences of other chemokine receptors and known GPCRs to identify the residues important for receptor activation. Residues at the extracellular surface facing into the binding pocket were implicated in signal transduction. These residues were then mutated to alanines to establish their role in ligand binding, receptor activation and chemotaxis of cell lines transfected with CCR7. Apart from identifying the residues critical for receptor activation, these studies also showed that the sites on CCR7 involved in the binding of CCL19 were very different from those responsible for the binding of CCL21 (Ott et al. 2004 b).

CCL21 is significantly expressed by high endothelial venules [HEVs] in the lymph nodes and at relatively lower levels by a small fraction of stromal cells in the T cell zones of lymph nodes, spleen and Peyer's patches (Hromas et al. 1997; Gunn et al. 1998). CCL19 is expressed by a subset of DCs and stromal cells in the T cell areas of the secondary lymphoid tissues (Ngo et al. 1998). Both CCL19 and CCL21 are potent attractants of T lymphocytes while CCL21 also weakly attracts B cells (Campbell et al. 1998; Ngo et al. 1998). These findings have led to the theory that CCL21 enables the recruitment of naïve T cells across HEVs into the lymph node while CCL19 acts in concert with CCL21 in concentrating them in the T zones and promoting encounter between T zone DCs and T cells (Campbell et al. 1998; Ngo et al. 1998).

The chromosomal locations of the genes encoding CCL19 and CCL21 have been mapped in mice, humans and chickens (Nagira et al. 1997; Yoshida et

al. 1997; Nakano and Gunn 2001; Wang et al. 2005). Both genes are adjacent to each other on mouse chromosome 4 in a region of conserved synteny to human chromosome 9p13 and chicken chromosome Z (Nagira et al. 1997; Yoshida et al. 1997; Nakano and Gunn 2001; Wang et al. 2005). These observations are consistent with the possibility that CCL19 and CCL21 arose from a common ancestral gene as a result of gene duplication during the course of evolution.

The genomic organization of CCL19 and CCL21 has been well characterized in the *plt* mice. These mice are homozygous for the paucity of lymph node T cell mutation (Gunn et al. 1999; Stein et al. 2000). The *plt* mice do not express CCL21 in the secondary lymphoid tissues and exhibit marked defects in leukocyte migration and immune responses (Gunn et al. 1999; Vassileva et al. 1999). The *plt* locus was mapped to mouse chromosome 4 on which CCL19 and CCL21 are located. Southern blot analysis and sequencing of genomic clones revealed that there are at least three CCL21 genes and one functional CCL19 gene in the mouse genome (Nakano and Gunn 2001). Sequence analysis indicated that the sequences of the CCL21 gene were highly conserved with minor deletions relative to the consensus sequence. Another important feature observed was that the CCL21*a* contained a serine residue at amino acid position 65 whereas the CCL21*b* and CCL21*c* forms contained a leucine residue at the same position (Nakano and Gunn 2001). The *plt* mice lack CCL21 serine in the lymphoid tissues and express CCL21 in the non-lymphoid tissues, as confirmed by northern blot analysis (Vassileva et al. 1999). Four CCL19 genes were identified in the mouse genome but three of them were pseudogenes and only one gene had the

methionine codon ATG, thus being translated into a functional CCL19 protein (Nakano and Gunn 2001).

1.12 Brief description of chicken chemokines

The chicken draft genome and the availability of the chicken EST database have facilitated the systematic identification, characterization and annotation of chemokine and chemokine receptor genes in the chicken genome (Wang et al. 2005; DeVries et al. 2006). About 23 chemokines encompassing the C, CC, CXC and CX_3C subfamilies and 14 chemokine receptors have been reported in the chicken (Hughes and Bumstead 2000; Sick et al. 2000; Hughes et al. 2001; Smith et al. 2004; Kaiser et al. 2005; Wang et al. 2005). The phylogenetic analyses suggested that most of the chicken chemokine and chemokine receptor genes shared a common ancestry with the mouse and human chemokine and chemokine receptor families. Also, there were significantly more gene duplications among the CC, CXC, CCR and CXCR subfamilies in mammals than in aves after their divergence (Kaiser et al. 1999; Hillier et al. 2004; Wang et al. 2005). Among the other birds, only three different chemokines namely CCL4, CCL5 and RANTES have been identified in ducks but not functionally characterized (Sreekumar et al. 2005). Comparison of the chromosomal segments containing chemokines in the human, mouse and chicken suggest that the genomic organization of chemokines is generally conserved between chickens and mammals. The chicken CC chemokines are located on chromosomes 1, 4, 6,

9, 13, 19 and Z. Chicken CCL19 and CCL21 are located on chromosome Z. In terms of gene structure, according to the chicken genome sequence, chicken chemokines share the characteristic three exon CC structures with mammals. Also, chicken chemokines are shorter in comparison to human counterparts due to the presence of shorter introns (Kaiser et al. 2005; Wang et al. 2005).

1.13 Functional role of CCL19

The biological role of CCL19 was established by the discovery of a spontaneous mutant mouse that was deficient in CCL19 and CCL21(Luther et al. 2000). This mutant was called *plt*, for paucity of lymph node T cells. These mice were shown to have greatly reduced numbers of migrating T cells into lymph nodes, Peyer's patches and the regions of lymphoid tissue in the small intestine (Nakano and Gunn 2001). Genetic mapping studies revealed that the *plt* locus in mice was located in the same region of chromosome 4 as the genes for CCL19 and CCL21. Previous studies have shown that the *plt* mice lack CCL21 serine and have only one functional CCL21 gene (Nakano and Gunn 2001). In an another independent attempt to extend these findings it was shown that these *plt* mice lacked the CCL19 gene also, as determined by Southern blot analysis (Luther et al. 2000). The extracts from the spleen and LN of *plt* mice were examined for the presence of CCL19 and CCL21 protein by a Western blot analysis using goat anti-mouse CCL19 or CCL21. CCL19 and CCL21 were undetectable in these extracts confirming that the *plt* mice were double deficient for CCL19 and CCL21 in the lymphoid tissues (Luther et al. 2000).

A successful adaptive immune response is initiated when antigen specific naïve T cells interact with antigen bearing mature DCs in the secondary lymphoid tissues, spleen and lymph node (Cyster 2000). As a consequence of DC activation, there is an upregulation of co-stimulatory molecules which thereby deliver an amplified signal to the T cells (Yanagihara et al. 1998). Activated DCs also trigger the release of specific cytokines and chemokines which polarize the subsequent T cell responses. The activated T cells then proliferate and migrate to the infected tissues to perform their effector functions (Gunn 2003). Thus, colocalization of naïve T cells and DCs in the peripheral lymphoid organs is key to the initiation of an antigen specific adaptive immune response (Kaiser et al. 2005; Marsland et al. 2005).

CCL19 is a potent leukocyte chemoattractant promoting the migration of antigen presenting cells [APCs] and lymphocytes to the secondary lymphoid organs in inflammatory immune responses (Yoshida et al. 1998; Marsland et al. 2005). The functional receptor for CCL19 is CCR7 (Yoshida et al. 1998). Upon antigen uptake by immature DCs followed by their subsequent activation by recognition of pathogen associated molecular patterns [PAMPs], CCR7 expression is upregulated which thereby directs the activated DCs into the T cell areas of the draining lymph node (Dieu et al. 1998; Sozzani et al. 1998; Yanagihara et al. 1998). This has been further substantiated by experimental observations in CCR7 deficient mice. In these mice, skin activated DCs show impaired migratory abilities to the draining LNs (Forster et al. 1999). CCL19 has also been identified as a potent inducer of DC maturation triggering the

upregulation of co-stimulatory molecules and involved in the release of pro-inflammatory cytokines such as IL-12 and TNF- α. The critical role of CCL19 and/or CCL21 as initiators of DC maturation is established by the observation that DCs in *plt* mice fail to mature completely after peripheral stimulation with TLR ligands and reach the LNs in a semi-mature state (Marsland et al. 2005). Thus CCL19 and CCL21 play pivotal roles in driving the maturation of PAMP-licensed DCs. These CCL19 and CCL21 activated DCs effectively differentiate thereby triggering the secretion of pro-inflammatory cytokines that determine the direction of subsequent T cell responses. Specifically, these cytokines are potent in inducing cytotoxic T cell and Th-1 responses (Marsland et al. 2005). This pro-inflammatory role of CCL19 is key to initiating effective, sustained cell mediated responses against viruses. In addition, CCL19 performs homeostatic functions regulating the transmigration of circulating lymphocytes across the HEVs into the lymph node (Baekkevold et al. 2001). *In vitro*, CCL19 is a potent inducer of both naïve lymphocytes and DCs as determined by transwell chemotaxis assays (Yoshida et al. 1997; Ngo et al. 1998; Kellermann et al. 1999).

1.14 CCL19 as a vaccine adjuvant

CCL19 is a leukocyte chemoattractant promoting the recruitment of DCs and lymphocytes to the secondary lymphoid tissues in inflammatory immune responses (Yoshida et al. 1998). CCR7, the functional receptor for CCL19 is upregulated on activated DCs and promotes their migration to the lymph node in response to CCL19 (Yanagihara et al. 1998; Marsland et al. 2005). CCL19 directs

the migration of mature DCs to the distinct T cell zones in the lymphoid organs thereby establishing a functional microenvironment to prime näive T cells (Yoshida et al. 1998; Marsland et al. 2005). In this regard, it has also been shown that due to CCL19 secretion, activated DCs are able to induce a polarized and motile state in naïve T cells. In a recent study, a video microscopy imaging system has been used to capture some of the early interactions between mature DCs and naïve T cells. Human naïve T cells were incubated along with autologous monocyte derived DCs. The response of naïve T cells to immature and mature DCs was observed and compared using video microscopy imaging. It was found that about 60% of naïve T cells became polarized in the presence of mature, activated DCs. They exhibited distinct morphological changes in comparison to naïve T cells cultured in the presence of immature DCs. These T cells were torpedo shaped containing a leading edge and a trailing uropod, which are characteristic of cells undergoing migration. Neutralizing antibodies to CCL19 and CCR7 resulted in approximately 92% reduction in the polarization of T cell responses. These findings conclusively support the theory that CCL19 facilitates interactions between naïve T cells and cognate Ag bearing APCs (Kaiser et al. 2005).

Manipulating the immune regulatory capacities of APCs using chemokines as immune modulators has scope in vaccine design and testing of antiviral therapies. In this regard, CCR7 ligands have demonstrated potential in orchestrating an effective antiviral immune response against HSV in mice (Eo et al. 2001; Toka et al. 2003). DCs, by virtue of their ability to activate näive T cells,

qualify as key players for the establishment of specific antiviral immunity. Antigen specific interactions between DCs and T cells leads to the production of antigen specific CTLs. The virus specific CTLs mediate viral clearance by the activation of antiviral pathways (Menne and Tennant 1999). Therefore, vaccines aimed at targeting specific antigens to DCs and polarizing the subsequent T cell responses provide protection against the particular pathogen (Figdor et al. 2004).

Experimental evidence shows that immunizing transgenic mice with hepatitis B surface antigens [HBsAg] by cytokine activated DCs can break tolerance and trigger an HBV specific CTL response (Shimizu et al. 1998; Engler et al. 2001). Cytokine activated, bone marrow derived DCs and freshly isolated splenic DCs from transgenic and non-transgenic mice were compared in their ability to process and present the HBsAg. This was done by measuring the susceptibility of HBsAg pulsed DCs to cytolysis. Transgenic mice infused with *ex-vivo* activated DCs produced normal numbers of HBsAg specific CTLs. The *ex-vivo* activated DCs delivered efficient co-stimulatory signals to the anergic, transgenic CTLs which enabled them to mount a sustained, HBsAg specific CTL response (Shimizu et al. 1998).

In another experiment, adoptive transfer of DCs pulsed with human papilloma virus [HPV] peptide induced HPV specific CTL responses, and protected mice from HPV induced tumors (Ludewig 2003). This indicated the involvement of DCs in the activation of anti-HPV T cells *in vivo*.

Most DC based vaccine strategies require that the DCs be customized *in vitro*, which involves manipulation of their maturation state. Upon interaction

with antigens, the DCs migrate to the draining lymph nodes to meet the T cells. The activated DCs secrete chemokines to which appropriate receptor bearing cells respond (Ngo et al. 1998). This is important to prime naïve T cells and initiate an antigen specific immune response. In mice, the migratory path of DCs responding to an artificial gradient of CCL19 has been tracked from the epidermis to the lymph nodes. These migratory DCs have then been targeted *in situ* and shown to establish effective antitumor immunity, upon loading of the specific tumor associated antigens [TAAs] (Kumamoto et al. 2002).

1.15 The DHBV model

Ducks are natural hosts of an important virus duck hepatitis B virus [DHBV] that is very similar to the HBV infecting humans (Menne and Tennant 1999). The hepadnaviruses have a very limited range of hosts comprising humans, woodchucks and ducks. Hence, the study of DHBV is very relevant to human health. The DHBV belongs to a family of hepadnaviruses which replicate in the liver of the host (Schultz and Chisari 1999; Tang and McLachlan 2002). The immune response to hepadnavirus encoded antigens has variable outcomes ranging from viral clearance to chronic infection (Chisari and Ferrari 1995; Guidotti et al. 1996). In patients infected with HBV, elimination of the virus is associated with the development of a vigorous cell mediated immune response (Guidotti and Chisari 1996; Guidotti et al. 1996). This involves recognition of viral epitopes in the context of class I MHC molecules by the HBV specific cytotoxic T lymphocytes [CTLs] resulting in the destruction of infected

hepatocytes (Guidotti and Chisari 1996; Guidotti et al. 1996; Menne and Tennant 1999). The activated CTLs kill the infected hepatocytes by inducing apoptosis and also release inflammatory cytokines IFN-γ and TNF- α. These cytokines control the viral infection at different levels. They recruit macrophages, NK cells and T cells to perform their effector functions. They upregulate antigen processing, transport and expression of MHC in the infected cells (Guidotti and Chisari 2001). In HBV transgenic mice, the role of these cytokines has been well established. Studies in these mice have revealed that antiviral mechanisms mediated by IFN-γ and TNF-α expel the virus from infected cells without damaging host infected organs [non-cytopathic] (Guidotti et al. 1999; Sette et al. 2001; Thimme et al. 2003). They activate antiviral pathways in hepatocytes, inhibiting replication and viral gene expression. The inability to mount an effective CTL response results in viral persistence due to incomplete viral clearance leading to the development of chronic infection.

Ducks make defective, truncated IgY antibodies (Magor et al. 1992; Magor et al. 1994) and mount a weak humoral response against viruses. The truncated antibodies lack the Fc fragment and are incapable of binding to macrophages or other APCs to perform effector functions. Hence ducks may rely mainly on the cellular arm of the immune system and the innate immune system for antiviral immunity. In the current scenario, given the narrow host range for hepadnaviruses, ducks are a valuable model to understand HBV disease pathogenesis and for the development of HBV specific antiviral strategies (Menne

and Tennant 1999; Mangisa et al. 2004). Identification of novel chemokines and cytokines in ducks would enable efficient use of the DHBV model.

1.16 Hypothesis and rationale

Our interest is in understanding the duck model of hepatitis B for evaluation of antiviral therapies. Exploiting the duck model of hepatitis B for design of antiviral therapies requires development of specific immunological reagents.

To identify immunologically relevant genes in the duck, we constructed a spleen cDNA library in an EST project. We isolated a unique EST 4B6 bearing homology to CC chemokine CCL19. Our hypothesis was that EST 4B6 could be the duck homologue of CCL19. In order to test this hypothesis we have characterized CCL19 and shown that it is chemotactic for duck PBMCs *in vitro* thereby suggesting its functional significance.

Chemokines are a family of proinflammatory peptides which have immense scope in vaccine design. They direct APCs and T cells to the lymph node during inflammatory immune responses. Manipulating the immune regulatory capacities of DCs using chemokines can help activate DCs and break tolerance, thereby skewing subsequent T cell responses and establishing an effective, sustained antigen specific antiviral immunity.

In this regard, using chemokines to manipulate dendritic cells *in vivo* would enable us to prime naïve T cells for a DHBV antigen specific immune response in ducks. We have identified and characterized the duck homologue of

CCL19 which is a dendritic cell chemokine that recruits DCs and naïve T cells to the lymph node. In mice, the critical role of CCL19 in DC maturation was established by the observation that DCs in *plt* mice fail to mature completely after peripheral stimulation with TLR ligands and reach the LNs in a semi-mature state (Marsland et al. 2005). Thus CCL19 plays a key role in driving the maturation of PAMP-licensed DCs. These CCL19 activated DCs effectively differentiate thereby triggering the secretion of pro-inflammatory cytokines that determine the direction of subsequent T cell responses. Specifically, these cytokines are potent in inducing cytotoxic T cell and Th-1 responses (Marsland et al. 2005). This pro-inflammatory role of CCL19 has implications in initiating effective, sustained cell mediated immune responses against viruses. Thus, CCL19 is an effective tool to modulate the immune regulatory capacities of DCs which thereby polarize subsequent T cell responses and establish an effective, antigen specific antiviral immunity.

We have demonstrated the function of recombinant CCL19 *in vitro* by chemotaxis assays thereby implying that it has potential as an immune modulator. These findings can be extended for design of therapeutic strategies against the DHBV thereby enabling efficient use of the DHBV model. One of the current limitations in maintaining protective long term immunity against HBV is the inability to eliminate cccDNA from infected hepatocytes. Targeting the DHBV using molecular adjuvants like CCL19 has potential in establishing DHBV antigen specific antiviral immunity.

2. MATERIALS AND METHODS

2.1 Ducks and duck PBMCs

A white Pekin duck [*Anas platyrhynchos*] was euthanized with Euthanyl. The peripheral blood was collected by cardiac puncture in blood collection tubes [BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ] containing sodium heparin. The blood was diluted with an equal amount of RPMI1640 media [GIBCO ^{TM}-Invitrogen Corporation] containing 5% heat inactivated fetal bovine serum [FBS]. The diluted blood samples [25 mls] were layered onto 15 mls of endotoxin tested Ficoll-Paque ^{TM} Plus [Amersham Biosciences, Uppsala, Sweden] and centrifuged at 274xg for 20 minutes at room temperature. The peripheral blood mononuclear cells [PBMCs] separated by density gradient centrifugation were collected and washed three times with RPMI1640 media. Viable cells were counted using Trypan Blue exclusion and a hemocytometer and diluted to obtain a final concentration of 10^6 cells/ml.

2.2 Clone identification and sequence analysis

Three thousand clones were randomly picked and sequenced from a spleen cDNA library in an EST project in our laboratory to identify immunologically relevant genes in the duck. One clone bearing significant homology to mammalian CC chemokine CCL19 was identified. The entire insert of 1320 bp was sequenced in both directions on an ABI Prism 377 automated DNA

sequencer [PE Applied Biosystems]. The primers used for sequencing are shown in Table 2. The chromatogram editing, nucleotide alignment and primer design was performed using the program GeneTool [version-2, BioTools, Edmonton, Alta]. Protein alignment and analysis of amino acid sequences was done using PepTool [BioTools].

Table 2. Primers used for sequencing EST 4B6

Primer	Seq 5' to 3'	Tm	Location of the primer
CCL19F1	CGGTGGCAACAACGTCCTGGA	66.47	CCL19 forward exon 1
CCL19F2	GCCGACTTGCTTCCACCTCCT	66.47	CCL19 forward 3' UTR
CCL19R2	CTGAGAGCTGGCCCCGATATG	66.47	CCL19 reverse exon 1
CCL19F3	CCCTCCACCATTCCCATCCTG	66.47	CCL19 forward 3' UTR
CCL19R3	TGCCCTCCTTTTCAGTCCCCA	64.52	CCL19 reverse 3' UTR

2.3 CCL19 probe preparation

To create a probe for northern and Southern hybridization analysis, a 230 bp product was amplified from the CCL19 cDNA sequence using forward primer CCL19F1 and reverse primer CCL19R2 that annealed to the exon 1 region [predicted based on the human CCL19 genomic sequence]. PCR amplification was done in a GeneAmp PCR system 9700 [PE Applied Biosystems, Foster City, Calif.] for 30 cycles using *Taq* polymerase [Qiagen, Mississauga, Ontario]. The CCL19 probe was radiolabelled with [^{32}P] α-dCTP by random priming [Prime-It

Random Primer Labeling Kit, Stratagene, TX] for detection in northern and Southern hybridizations.

2.4 Northern hybridization

Total RNA samples were isolated from different tissues including heart, kidney, lung, duodenum, brain, spleen, liver and bursa using TriZol [InVitrogen, life technologies, Carlsbad, CA, USA] according to the manufacturer's instructions. About 10μg of RNA samples from the various tissues were run on a 1.2% agarose, 0.6% formaldehyde gel and blotted onto a Nytran Supercharge nylon transfer membrane and UV cross-linked [UV Stratalinker 2400, Stratagene]. The blot was prehybridized at 42^{0}C for 2 hours and hybridized overnight with [^{32}P] labelled CCL19 probe in formamide hybridization solution [50% formamide, 5x Denhardt's reagent, 4x SSPE and 1% SDS]. The blot was washed at high stringency in 0.1x SSPE, 0.1%SDS at 65^{0}C and exposed to Kodak BioMax XAR film at –80^{0}C for one week.

2.5 Southern hybridization

High molecular weight genomic DNA obtained from erythrocytes of a White Pekin duck was digested to completion with restriction enzymes BamHI, EcoRI, HindIII and PstI and separated on 0.8% agarose gels, blotted onto a Nytran Supercharge nylon transfer membrane and immobilized by UV cross-linking. The blot was prehybridized at 42^{0}C for 6 hours and hybridized overnight with [^{32}P] labelled CCL19 probe in formamide hybridization solution [50%

formamide, 5x Denhardt's reagent, 5% dextran sulfate, 4x SSPE and 1% SDS]. The blot was washed at low stringency in 1x SSPE, 0.1%SDS at 52^{0}C and exposed to Kodak BioMax XAR film at –80^{0}C for a week.

2.6 Production and purification of recombinant CCL19

The sequence encoding amino acids 22-99 encompassing the entire mature protein was amplified by PCR using the forward primer CCL19F.ex 5'-CATATGGGTGGCAACAACGTCCTGGA-3' and reverse primer CCL19R.ex 5'-CTCGAGATTGCCTTTATTTGGGACCTTC-3', purified using QIAquick PCR purification Kit [QIAGEN, Maryland, USA]. The vector was digested with the restriction enzymes NdeI and XhoI. The purified PCR product was subcloned into the NdeI and XhoI sites of the pET29b vector [Novagen, Inc., Madison, WI] in frame with the COOH-terminal 6xHis tag and BL21(DE3) cells were transformed. Briefly, a 50 ml overnight culture in LB plus 30μg/ml kanamycin was inoculated into one litre LB containing 30μg/ml kanamycin at 37^{0}C. After 1-2 hours of incubation, at an O.D. $_{600}$ of 0.6, 1 mM IPTG was added to the culture and incubated overnight. Bacteria were harvested by centrifugation at 3000xg for 20 minutes at 4^{0}C. The pellet was resuspended in 10mM Tris-HCl pH 8, 10mM EDTA, sonicated, washed and centrifuged three times at 3000xg for 20 minutes at 4^{0}C to recover the inclusion bodies containing recombinant CCL19. The inclusion bodies were lysed by sonication in cell lysis buffer [8M Urea, 1% Triton-X 100, 50mM Tris-HCl, 10mM EDTA, lysozyme 0.1μg/ml and 0.1mM PMSF]. The lysate was cleared by centrifugation, run on a NiNTA agarose column [QIAGEN,

Mississauga, Ontario] and eluted in 8M urea buffer containing 300mM imidazole to obtain pure, recombinant CCL19. The purity of recombinant CCL19 was assessed on a 15% reducing SDS polyacrylamide gel stained with Coomassie Blue. Recombinant CCL19 was detected as a band at the expected size of 11-12 kDa. The protein fragments were transferred to nitrocellulose membranes [Bio-Rad Laboratories, CA, USA], blocked with 0.5% BSA for 30 minutes, washed in TTBS and detected by chemiluminescence using an antibody specific for the 6x His tag [ECL™ Western Blotting Kit, Amersham Biosciences, Buckinghamshire, UK]. The urea was removed by dialysis in a stepwise manner from a concentration of 8M to 1M. The concentration of purified recombinant CCL19 in 1M urea buffer was estimated with a Micro BCA ™ protein assay kit [Pierce, IL, USA].

2.7 Chemotaxis assays

Chemotaxis assays were performed using 24 well homemade chemotaxis chambers. The pure, recombinant CCL19 in 1M urea was diluted with RPMI-1640 media containing 5% heat inactivated FBS to obtain desired concentrations. The diluted chemokine solution was used to fill the bottom chamber. A polycarbonate membrane filter with a pore size 5μm in diameter, [Neuroprobe Inc, Gaithersburg, MD, USA] was placed over the bottom chamber using a pair of sterile foreceps. The top chamber was then screwed in place on top of the membrane filter. Cell suspension containing approximately $1.5x10^5$ cells was placed in the top chamber. The chemotaxis chambers were incubated for 4 hours

in 5% CO_2 at 37^{0}C and cells were allowed to migrate to the lower chamber in the presence or absence of the chemokine. The cells in the top chamber that did not migrate in response to the chemokine were removed. The top chamber was rinsed two times in sterile PBS [pH 7.2] gently, without damaging the membrane filter. The top chamber was then unscrewed. The membrane filter was carefully removed using foreceps and placed upside down on a glass slide. The filter carrying cells that migrated through the pores to the underside of the membrane filter were fixed in Hema-3 fixative [Fisher Scientific, Kalamazoo, MI, USA], air dried briefly and stained using Hema-3 stain [Fisher Scientific, Kalamazoo, MI, USA]. Cells in 5 different fields were then counted at 400X magnification using a compound microscope. Cells that migrated in response to the media alone [without recombinant CCL19] served as the negative control.

To demonstrate the specific chemotactic ability of recombinant CCL19, chemotaxis assays were set up simultaneously with equal concentrations of purified, recombinant CCL19 and purified, recombinant β_2 microglobulin expressed in *E. coli*. The subcloning, protein expression and protein purification of β_2 microglobulin was done by Debra Moon. Cells that migrated in response to recombinant CCL19 and recombinant β_2 microglobulin were enumerated similarly as mentioned before. To eliminate the possibility of contamination with bacterial LPS, 10 μg/ml of recombinant CCL19 and recombinant β_2 microglobulin were incubated with 10 μg/ml of Polymyxin B sulfate [Sigma] at 4^{0}C for 1 hour. Polymyxin B sulfate binds to bacterial LPS in solution. Chemotaxis assays were performed with recombinant CCL19 and recombinant β_2

microglobulin treated with or without Polymyxin B sulfate to demonstrate that migration is not due to contaminating LPS from *E. coli*.

2.8 Quantification of LPS using limulus amoebocyte lysate test

The amount of contaminating LPS in recombinant CCL19 and recombinant β_2 microglobulin was quantified using the limulus amoebocyte lysate endosafe kit according to the manufacturer's instructions [Charles River]. Briefly, 200 µl of recombinant CCL19 or recombinant β_2 microglobulin in 1M urea was incubated in the single test vial containing buffered lysate at 37^0C for an hour. *Salmonella typhimurium* at a concentration of 10µg was used as a positive control for detection of LPS. Sterile pyrogen free water was used as the negative control. A positive result was indicated in *Salmonella typhimurium* by the formation of a firm gel capable of maintaining its integrity when inverted at 180^0. A negative test was characterized by the absence of gel or by the formation of a viscous mass. Both recombinant CCL19 and recombinant β_2 microglobulin gave a negative result indicating that the amount of contaminating LPS was below the detection limit of the endosafe reagent [0.25EU/ml].

3. RESULTS

3.1 Identification of a duck homologue of CCL19

To enable identification of immunologicaly relevant genes in the duck *[Anas platyrhynchos]*, 3000 randomly selected clones from a duck spleen cDNA library were sequenced. We identified a unique clone 4B6 [GenBank accession number - AY682098] bearing significant homology to the mouse and human CC chemokine CCL19 [Fig. 2]. The full length cDNA was 1320 base pairs in length and contained an open reading frame starting with the methionine codon that encoded a polypeptide of 99 amino acids with a predicted molecular weight of 12 kDa. The 3' noncoding region was longer than that of the mammalian CCL19 and featured a typical AATAAA polyadenylation signal sequence [Fig. 2].

The deduced polypeptide sequence consisted of a highly hydrophobic amino terminal region characteristic of a signal peptide with a predicted cleavage site between amino acid residues Gly-22 and Gly-23, based on the mouse and human CCL19 polypeptide sequences [Fig. 2]. The predicted mature protein had an estimated isoelectric point of 11.04 [www.expasy.org/tools/pi_tool.html]. The highly basic polypeptide of 99 amino acids contained 16 positively charged amino acid residues and 7 negatively charged amino acid residues throughout the entire sequence. There were no potential N glycosylation sites.

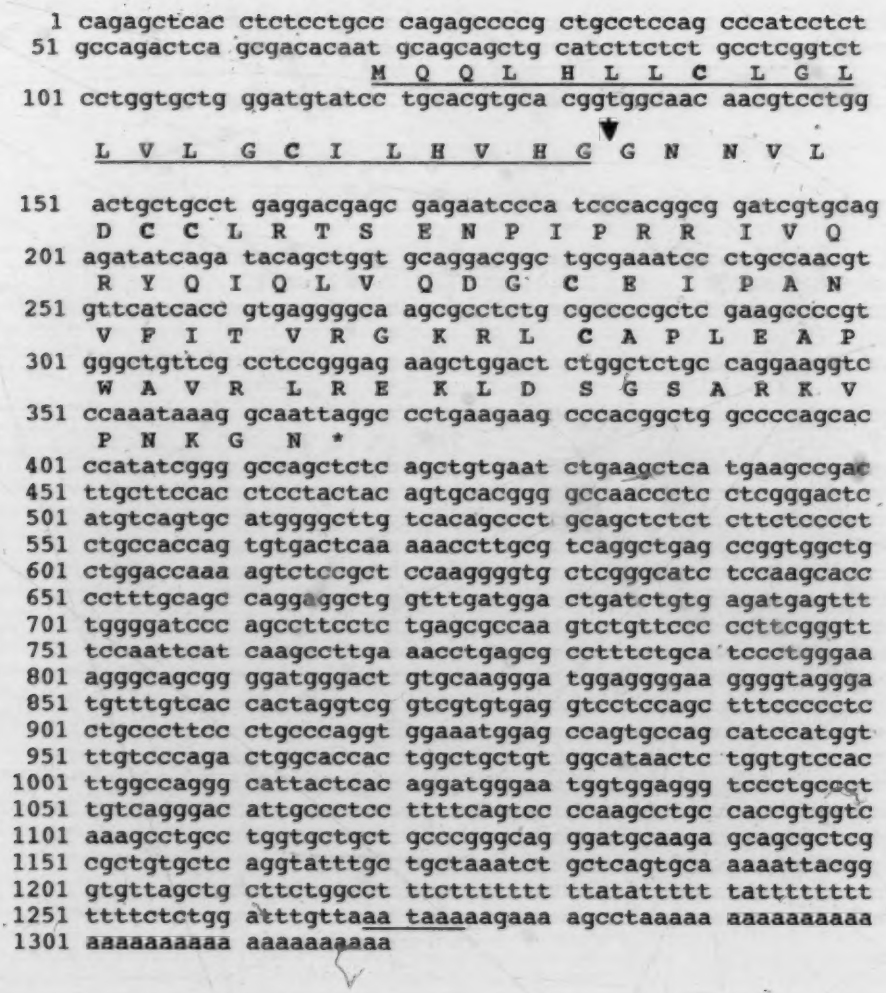
1 cagagctcac ctctcctgcc cagagccccg ctgcctccag cccatcctct
51 gccagactca gcgacacaat gcagcagctg catcttctct gcctcggtct
M Q Q L H L L C L G L
101 cctggtgctg ggatgtatcc tgcacgtgca cggtggcaac aacgtcctgg
L V L G C I L H V H G G N N V L
151 actgctgcct gaggacgagc gagaatccca tcccacggcg gatcgtgcag
D C C L R T S E N P I P R R I V Q
201 agatatcaga tacagctggt gcaggacggc tgcgaaatcc ctgccaacgt
R Y Q I Q L V Q D G C E I P A N
251 gttcatcacc gtgaggggca agcgcctctg cgccccgctc gaagccccgt
V F I T V R G K R L C A P L E A P
301 gggctgttcg cctccgggag aagctggact ctggctctgc caggaaggtc
W A V R L R E K L D S G S A R K V
351 ccaaataaag gcaattaggc cctgaagaag cccacggctg gccccagcac
P N K G N *
401 ccatatcggg gccagctctc agctgtgaat ctgaagctca tgaagccgac
451 ttgcttccac ctcctactac agtgcacggg gccaaccctc ctcgggactc
501 atgtcagtgc atggggcttg tcacagccct gcagctctct cttctccct
551 ctgccaccag tgtgactcaa aaaccttgcg tcaggctgag ccggtggctg
601 ctggaccaaa agtctccgct ccaaggggtg ctcgggcatc tccaagcacc
651 cctttgcagc caggaggctg gtttgatgga ctgatctgtg agatgagttt
701 tggggatccc agccttcctc tgagcgccaa gtctgttccc ccttcgggtt
751 tccaattcat caagccttga aacctgagcg cctttctgca tccctgggaa
801 agggcagcgg ggatgggact gtgcaaggga tggaggggaa ggggtaggga
851 tgtttgtcac cactaggtcg gtcgtgtgag gtcctccagc tttccccctc
901 ctgcccttcc ctgcccaggt ggaaatggag ccagtgccag catccatggt
951 ttgtcccaga ctggcaccac tggctgctgt ggcataactc tggtgtccac
1001 ttggccaggg cattactcac aggatgggaa tggtggaggg tccctgccct
1051 tgtcagggac attgccctcc ttttcagtcc ccaagcctgc caccgtggtc
1101 aaagcctgcc tggtgctgct gcccgggcag ggatgcaaga gcagcgctcg
1151 cgctgtgctc aggtatttgc tgctaaatct gctcagtgca aaaattacgg
1201 gtgttagctg cttctggcct ttctttttt ttatattttt tatttttttt
1251 ttttctctgg atttgttaaa taaaaagaaa agcctaaaaa aaaaaaaaaa
1301 aaaaaaaaaa aaaaaaaaaa

Fig. 2. Full length nucleotide and deduced amino acid sequence of duck CC chemokine CCL19. The predicted signal sequence and polyadenylation signal sequence is underlined. The arrow indicates the cleavage site of the signal sequence. Cysteine residues are shown in bold. The sequence data is available in GenBank under the accession number AY682098.

The full length polypeptide sequence encoded by 4B6 showed significant homology to other vertebrate CC chemokines [Fig. 3]. The four cysteine residues in the mature protein were highly conserved as in the CC chemokine subfamily in all vertebrate species. The protein encoded by our duck clone 4B6 shared 78% identity to the chicken CCL19, 46% identity to the mouse CCL19 and 44% identity to the human CCL19 at the amino acid level [GenBank accession numbers - AF059208, AJ223410].

To investigate the evolutionary relationship between the closely related vertebrate CC chemokines CCL19 and CCL21, a phylogenetic tree was drawn from a Clustal W generated amino acid alignment of the available vertebrate CCL19 and CCL21 sequences [Fig. 4]. The duck CCL19 appears to be more closely related to chicken and mammalian CCL19 homologues than to chicken and mammalian CCL21 homologues.

To determine the tissue distribution pattern of duck CCL19 a northern blotting analysis was done. Eight different tissues from a duck including heart, kidney, lung, duodenum, brain, spleen, liver and bursa were hybridized with a probe for CCL19 [Fig. 5]. The duck CCL19 was found to be significantly expressed only in spleen and weakly expressed in the lung. The expression of CCL19 in the spleen is consistent with that of its mammalian counterparts [Fig. 5]. The blot was stripped and re-hybridized with GAPDH as a control for amounts of RNA loaded.

```
                                                                    %
Duck            --MQQLHLLCLGLLVLGCILH--VHGGNNVLDCCLRTSENPIPRRIVQRYQIQL
Chicken         --..R..V...S....R.V..--.YA.............K...W....D.RM..      78
Rat             MASRVTP..AFS....WTFSAPALG.A.DAE....SVTQR...GN..KAFRYL.      46
Mouse           MAPRVTP..AFS....WTFPAPTLG.A.DAE....SVTQR...GN..KAFRYL.      46
Dog             MAS.AAV..A.S..L.WTSPA--LG.A.DAE....SVTQR...GN..RAFHYL.      43
Human           ----MAL..A.S....WTSPAPTLS.T.DAE....SVTQK...GY..RNFHYL.      44
Rhesus monkey   ----MAL..A.S....WTSPAPTLS.T.DAE....SVTQK...GY..RNFRYL.      42

Duck            VQDGCEIPANVFITVRGKRLCAPLEAPWAVRLREKLDSGSARKVPNKGN--------
Chicken         .....D...T..I.AK.......PQ...VL.L.....TS.......Q..--------
Rat             I....RV..V..T.L..YQ.....DQ..VE.IIRR.KKS.SKAKAAAP--KGALCLE
Mouse           NE...RV..V..T.L..YQ....PDQ..VD.IIRR.KKS..KNKG.ST--RRSPVS-
Dog             IK...RL..V..T.L...Q....PDQ..VD.IIRR.QKN..KNKGHSS---------
Human           IK...RV..V..T.L...Q....PDQ..VE.IIQR.QRT..KMKRRSS---------
Rhesus monkey   IK...RV..V..T.L...Q....PDQ..VE.IIQR.QRT.TKMKRRSS---------
```

Sequence:1- Duck *(Anas platyrhynchos)* CCL19 (AY682098)
Sequence:2- Red jungle fowl *(Gallus gallus)* CCL19 (XM_424980).
Sequence:3- Rat *(Rattus norvegicus)* CCL19 (XM_342824).
Sequence:4- Mouse *(Mus musculus)* CCL19 (AF059208).
Sequence:5- Dog *(Canis familaris)* CCL19 (AB163919).
Sequence:6- Human *(Homo sapiens)* CCL19 (AJ223410).
Sequence:7- Rhesus monkey *(Macaca maculata)* CCL19 (AF449273).

Fig. 3. Amino acid alignment of duck CCL19 with other related CC chemokine sequences. The full length amino acid sequence of duck CCL19 is aligned with related CC chemokine sequences using the T-Coffee alignment program. The gene accession numbers are mentioned below the alignment. The identical amino acid residues are indicated by a dot. The percent identity to duck CCL19 is indicated on the right.

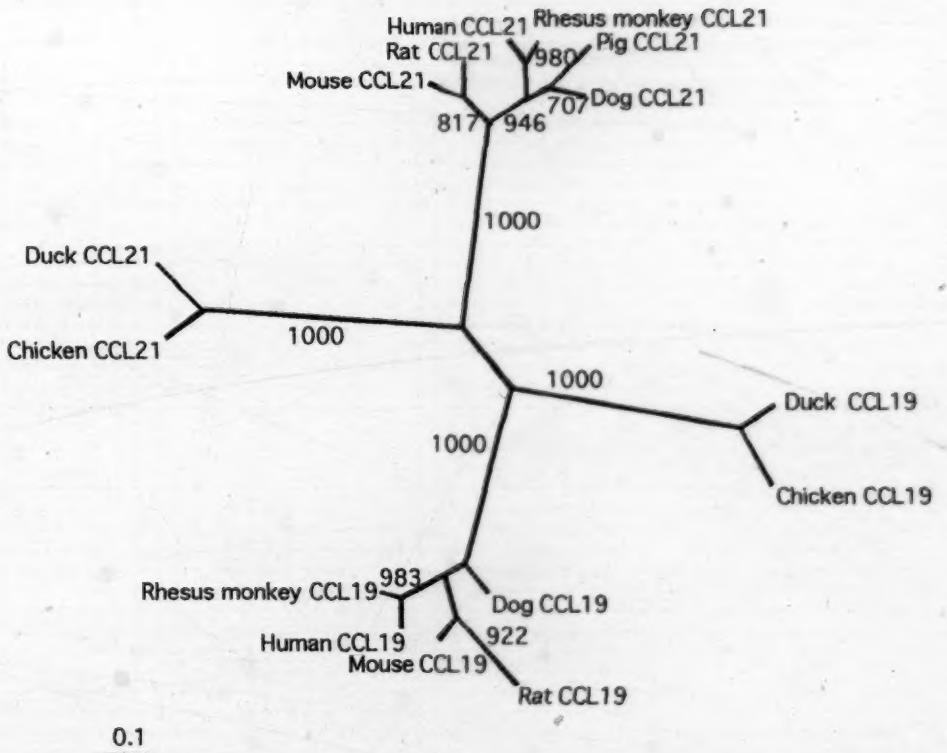

Fig. 4. Phylogenetic analysis showing the relationship between different vertebrate CCL19 and CCL21. The tree was constructed using the Clustal W program (www.ddbj.nig.ac.jp). The sequences encoding the mature protein of duck, chicken, mouse, human, rat, dog and rhesus monkey CCL19 (GenBank), duck CCL21 (unpublished), chicken CCL21 (U.D. Chick EST database), mouse, human, rat, dog, pig and rhesus monkey CCL21 were aligned using Neighbour-

joining method. The resultant trees were bootstrapped 1000 times and bootstrap values greater than 700 considered significant, are shown in the figure. The accession numbers for all the sequences are indicated below.

Duck *(Anas platyrhynchos)* CCL19 (AY682098).

Red jungle fowl *(Gallus gallus)* CCL19 (XM_424980).

Rat *(Rattus norvegicus)* CCL19 (XM_342824).

Mouse *(Mus musculus)* CCL19 (AF059208).

Dog *(Canis familaris)* CCL19 (AB163919).

Human *(Homo sapiens)* CCL19 (AJ223410).

Rhesus monkey *(Macaca maculata)* CCL19 (AF449273).

Duck *(Anas platyrhynchos)* CCL21 (unpublished).

Red jungle fowl *(Gallus gallus)* CCL21 (U. D. Chick EST database).

Rat *(Rattus norvegicus)* CCL21(AAH86571).

Mouse *(Mus musculus)* CCL21 (AF307987).

Dog *(Canis familaris)* CCL21(AB164433).

Human *(Homo sapiens)* CCL21 (AB002409).

Rhesus monkey *(Macaca maculata)* CCL21 (AF449275).

Pig CCL21 (AJ585194).

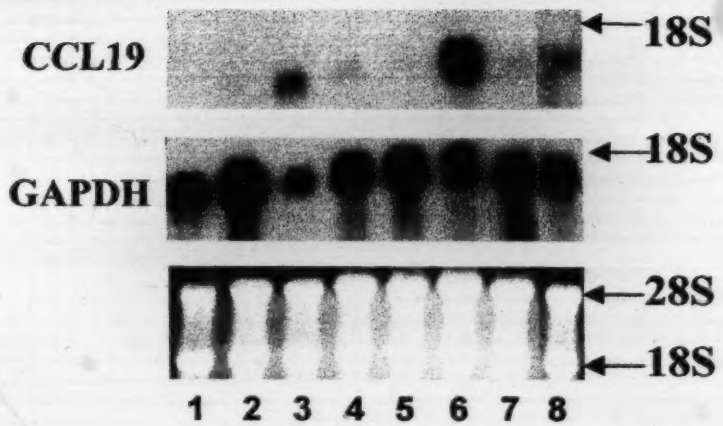

Fig. 5. Northern blot analysis showing CCL19 expression in various duck tissues. The northern blot made from total RNA samples isolated from eight different tissues in a duck was probed with ^{32}P labelled CCL19. Lane 1:Heart; 2:Kidney; 3:Lung; 4:Duodenum; 5:Brain; 6:Spleen; 7:Liver; 8:Bursa. *I:* Hybridization with CCL19 probe. *II:* Hybridization with GAPDH probe as a control for amounts of RNA loaded. *III:* RNA gel showing the different lanes as a control for quality of RNA loaded.

To establish gene copy number of CCL19 in the genome, Southern blot analysis was performed. Duck genomic DNA was digested with four different restriction enzymes including BamHI, EcoRI, HindIII, PstI and hybridized with a probe for CCL19. In each digest, there were two hybridizing restriction fragments for each of the different enzymes. There were no cut sites in the probe for any of the enzymes tested. This suggested that CCL19 probably existed in two copies in the duck genome [Fig. 6].

3.2 Production and purification of recombinant CCL19

The cDNA encoding the mature protein was directionally cloned into the NdeI and XhoI sites of the pET29b vector in frame with a 6X His-tag at the carboxyl terminus. The recombinant protein expressed in *E.coli* was detected by SDS-PAGE stained with Coomassie brilliant blue at the expected size of 12 kDa [Fig. 7]. Recombinant CCL19 purified on a nickel agarose [NiNTA] column under denaturing conditions with 8M urea was detected by SDS-PAGE st ed with Coomassie brilliant blue [Fig. 8] and confirmed by western blotting analysis using an antibody specific for the 6X His-tag [Fig. 9]. A significant fraction of recombinant CCL19 existed as monomers while a small percentage of the protein formed homodimers. The urea was removed by dialysis in a stepwise manner. Concentration of recombinant CCL19 in 1M urea buffer determined by a micro BCA assay was approximately 1 mg/ml.

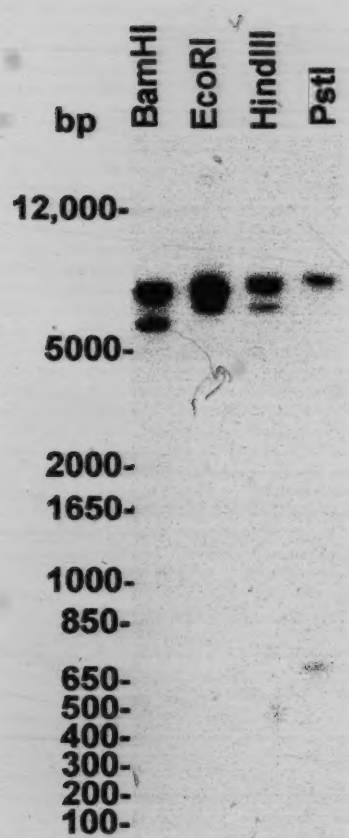

Fig. 6. Southern blot analysis indicating genomic organization of duck CCL19. Southern blot made from duck genomic DNA digested with four different restriction enzymes was hybridized with ^{32}P labelled CCL19 probe. The $1kb^{+}$ DNA size marker is indicated on the left.

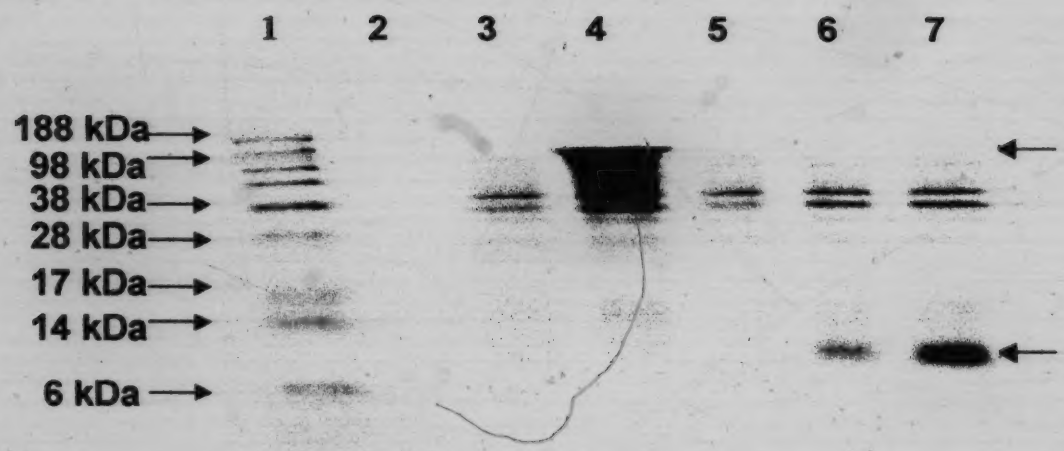

Fig. 7. SDS-PAGE analysis of recombinant CCL19. The *E. coli* culture BL21 [DE3] was induced for expression of 6X His-tagged recombinant CCL19. The different fractions collected at various time points were detected by SDS-PAGE stained with Coomassie brilliant blue. Lane 1: Molecular weight marker; 2: Empty; 3: Uninduced *E. coli* culture; 4: β–galactosidase expression 180 minutes after induction [control]; 5: Uninduced *E. coli* culture; 6: CCL19 expression 120 minutes after induction; 7: CCL19 expression 180 minutes after induction. The arrow at the top right indicates expected size of the control and the arrow at the bottom right shows the expression of CCL19.

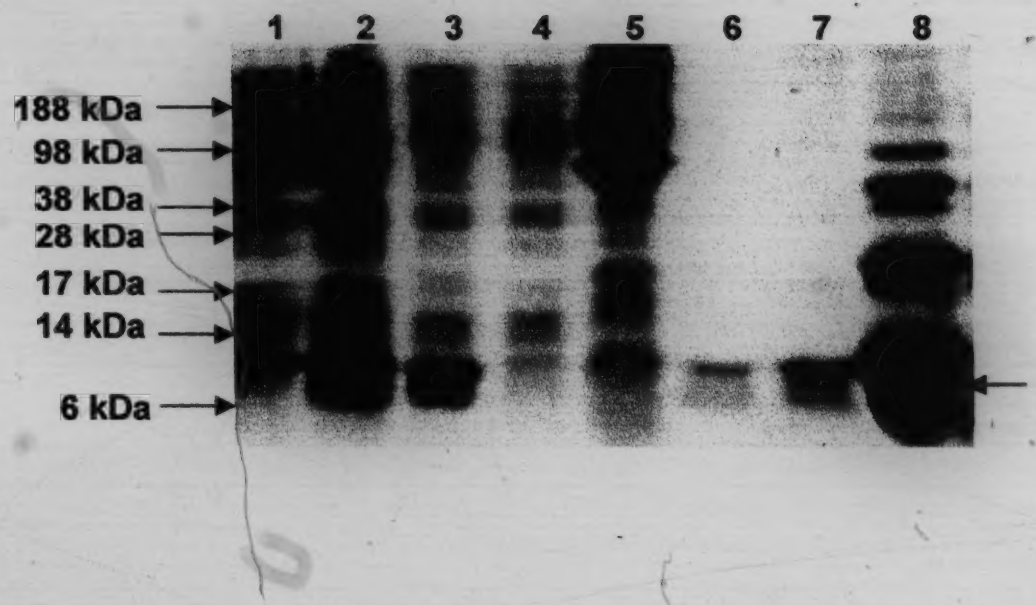

Fig. 8. SDS-PAGE analysis of affinity purified recombinant CCL19. The lysed inclusion bodies containing 6X His-tagged recombinant CCL19 was purified on a nickel agarose column. The various fractions collected during purification were detected by SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue. Lane 1: Uninduced *E. coli* culture; 2: CCL19 expression 180 minutes after induction; 3: Inclusion bodies; 4: Flow through; 5: Wash; 6-8: Elution fractions of purified recombinant CCL19. The molecular weight marker is indicated on the left. The arrow at the bottom right indicates purified CCL19 obtained in elution fraction 3.

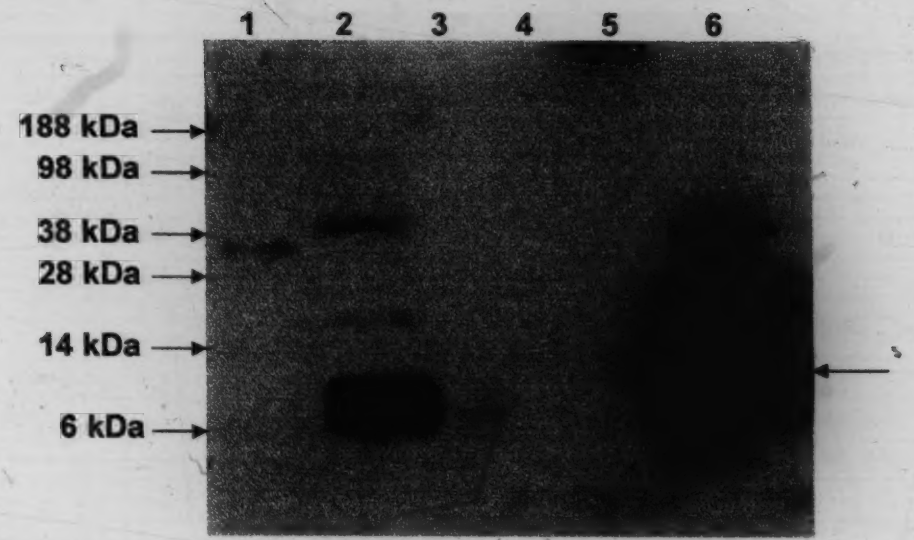

Fig. 9. Western blot analysis of affinity purified recombinant CCL19. The lysed inclusion bodies containing 6X His-tagged recombinant CCL19 was purified on a nickel agarose column. The various fractions collected during purification were detected on a western blot with an antibody specific for the 6X His tag. Lane 1: Positive control-goldfish macrophage receptor (M-CSF); 2: Inclusion bodies; 3: Flow through; 4: Wash 1; 5: Wash 2; 6: The arrow at the bottom right indicates purified recombinant CCL19. The molecular weight markers are indicated on the left.

3.3 Chemotactic activity of recombinant CCL19 on freshly isolated duck peripheral blood mononuclear cells (PBMCs)

To test the capability of recombinant CCL19 to attract duck PBMCs *in vitro*, a chemotaxis assay was set up with or without different concentrations of CCL19 as indicated [Fig. 10]. Cells migrating across the pores of the transwell membrane filter to the underside were counted and expressed as number of migrating cells per 5 different high power fields. Number of cells migrating in response to the media alone [without CCL19] served as the negative control. A significant dose response was observed with maximum number of PBMCs migrating towards the underside of the transwell membrane filter at a concentration of 10μg/ml [Fig. 10].

To demonstrate that migration towards recombinant CCL19 across the pores of the transwell membrane filter was directional and not random movement, migration assays were set up with recombinant CCL19 as a gradient [specific concentrations of CCL19 in the bottom chamber alone] or without a gradient [equal concentrations of CCL19 in the top and bottom chambers] [Fig. 11]. At all concentrations tested, migration of PBMCs towards CCL19 in a gradient was significantly higher than migration without a gradient indicating that the response to recombinant CCL19 was chemotactic [Fig. 11].

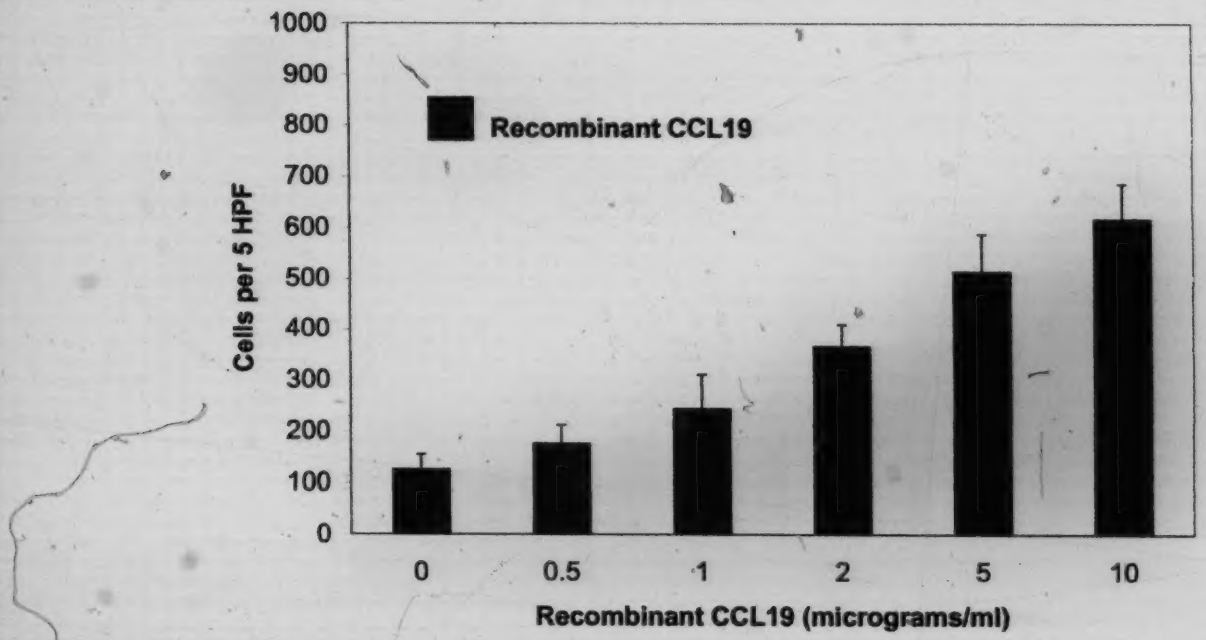

Fig. 10. Migration of duck PBMCs in response to recombinant CCL19. Chemotaxis assays showing the number of PBMCs counted in 5 high power fields to the underside of the transwell membrane filter in response to the specified concentrations of recombinant CCL19. Data indicated as mean ± standard error in 5 experiments using different individuals for each experiment.

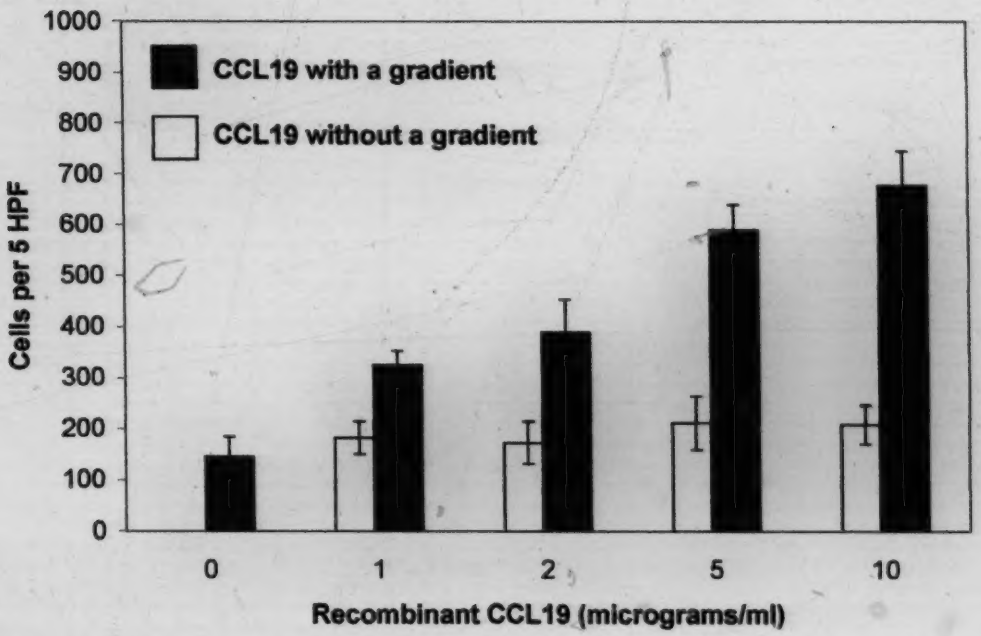

Fig. 11. Migration of duck PBMCs in the presence or absence of a gradient of recombinant CCL19. Chemotaxis assays showing the number of PBMCs counted in 5 high power fields that migrated to the underside of the transwell membrane filter in response to the specified concentrations of recombinant CCL19. Recombinant CCL19 was set up as a gradient (*gradient*-specified concentration of CCL19 in the lower chamber alone) or without a gradient (*no gradient*-equal concentrations of CCL19 in the upper and lower chambers). Data indicated as mean ± standard error in 5 experiments using different individuals for each experiment.

To assess the specific chemotactic ability of recombinant CCL19 to attract duck PBMCs, compared to any other recombinant protein expressed in *E. coli*, a chemotaxis assay was set up simultaneously with equal concentrations of recombinant CCL19 and recombinant β_2 microglobulin [Fig. 12]. The number of cells migrating in response to the two different proteins was determined. The number of PBMCs migrating in response to the media alone and the 1M urea buffer served as the negative control. The PBMCs demonstrated increased responsiveness to recombinant CCL19 than recombinant β_2 microglobulin at all concentrations tested indicating that the migration was CCL19 specific [Fig. 12].

To establish that migration was specific in response to recombinant CCL19 and not LPS from *E.coli*, chemotaxis assays were performed with recombinant CCL19 and recombinant β_2 microglobulin with or without Polymyxin B sulfate [Sigma] at a concentration of 10μg/ml [Fig. 13]. Polymyxin B sulfate binds to bacterial LPS in solution. A significant response of duck PBMCs to endotoxin treated recombinant CCL19 in comparison to untreated recombinant CCL19 confirmed that migration is not due to contaminating LPS from *E. coli* [Fig. 13].

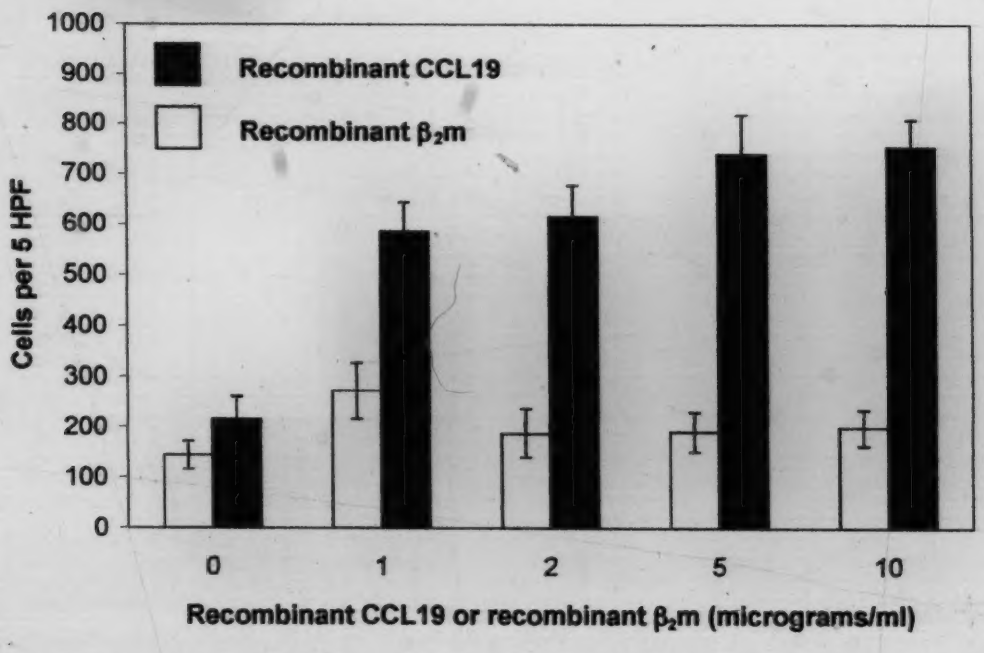

Fig. 12. Migration of duck PBMCs towards recombinant CCL19 or recombinant β_2 microglobulin. Chemotaxis assays showing the number of PBMCs counted in 5 high power fields on the underside of the transwell membrane filter that migrated in response to the specified concentrations of recombinant CCL19 or recombinant β_2 microglobulin. Data indicated as mean $\pm$ standard error in 5 experiments using different individuals for each experiment.

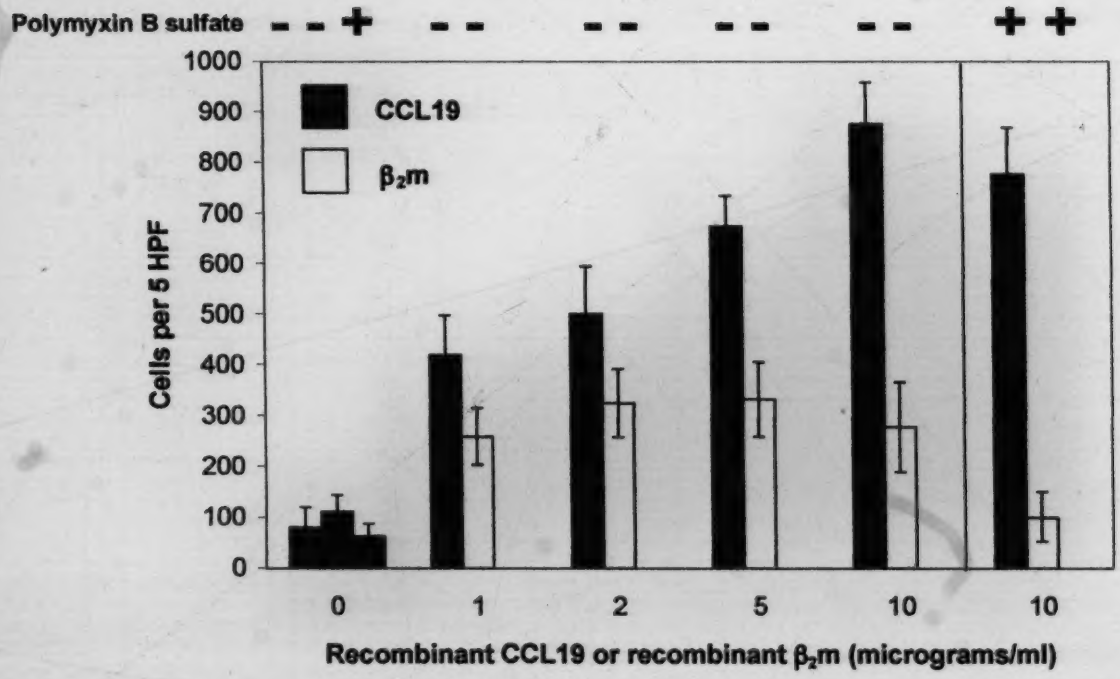

Fig. 13. Migration of duck PBMCs toward specified concentrations of recombinant CCL19 and recombinant β_2 microglobulin incubated with or without polymyxin B sulfate. Chemotaxis assays showing the number of PBMCs counted in 5 high power fields to the underside of the transwell membrane filter that migrated in response to the specified concentrations of recombinant CCL19 and recombinant β_2 microglobulin. The sample(s) in the third and last panel were treated with polymixin B sulfate. Data indicated as mean $\pm$ standard error of 5 experiments using different individuals in each experiment.

4. DISCUSSION

4.1 Characterization of a duck homologue of CCL19

In the present study, we have identified the duck homologue of CC chemokine CCL19 and demonstrated that it is chemotactic for peripheral blood mononuclear cells (PBMCs) *in vitro*. Random selection of 3000 clones from a duck *(Anas platyrhynchos)* spleen cDNA library and subsequent sequencing yielded a novel clone 4B6 identical to mammalian CCL19 homologues. Our findings based on sequence characteristics, high degree of sequence homology with other vertebrate CCL19 homologues and phylogenetic data strongly suggest that clone 4B6 is the probable duck homologue of CCL19. The full length cDNA encoded a highly basic polypeptide typical of all vertebrate CC chemokines (Imai et al. 1996; Nagira et al. 1997). Duck CCL19 shared homology with other members of the vertebrate CCL19 subfamily with approximately 40-78% amino acid identity across species. The four cysteine residues in the mature protein stabilized by the disulphide bonds are highly conserved which is characteristic of all vertebrate CC chemokines (Yoshida et al. 1997; Ngo et al. 1998; Basu et al. 2002). The high degree of conservation of the cysteine residues throughout evolution imply an important biological role for this motif in the structure. Also, the DCCL motif in the N terminus is highly conserved among the vertebrate CCL19 homologues suggesting its functional significance.

We eliminated the possibility that duck 4B6 encoded the closely related homologue of CCL19, another CC chemokine, CCL21. CCL19 lacks the additional highly basic C terminal domain found in CCL21. Chicken CCL21 is available as an EST in the U.D. (University of Delaware) Chick EST database (unpublished). We also identified duck CCL21 in the EST project from the spleen cDNA constructed in our lab (Jianguo Xia, unpublished data). An important characteristic feature of CCL21 is the presence of a longer carboxy-terminal domain with a high content of basic amino acids (Nagira et al. 1997). This C terminal region typical of CCL21 is highly conserved in both mice and humans (Hedrick and Zlotnik 1997; Hromas et al. 1997; Tanabe et al. 1997). Apart from the four cysteine residues found in the mature protein of all CC chemokines, the carboxyl terminus of CCL21 contains two additional cysteines (Hedrick and Zlotnik 1997; Hromas et al. 1997; Tanabe et al. 1997). The disulphide bridge formed between these two cysteines results in the formation of a C terminal structure unique to CCL21 (Hedrick and Zlotnik 1997; Hromas et al. 1997; Tanabe et al. 1997). Duck CCL21 contains this unique C terminal extension of basic amino acids while duck CCL19 lacks it.

The sequences in the N terminal domain of duck CCL19 in comparison to mammalian, duck and chicken CCL21 counterparts are fairly conserved. The N terminal residues preceding the first cysteine in the mature mammalian CCL19 like asparigine in the third position, aspartate in the fourth and seventh positions are important for ligand binding (Ott et al. 2004 a). Although the interactions responsible for ligand binding and receptor activation are independent of each

other, the N terminus is considered crucial for the biological activity of CCL19 (Ott et al. 2004). The N terminal region is conserved throughout phylogeny indicating the importance of this functional domain in CCL19.

The evolutionary relationship between CCL19 and CCL21 was established based on phylogenetic analysis. Regions encompassing the mature protein of available vertebrate CCL19 and CCL21 homologues were analyzed. Our data suggests that duck CCL19 is more closely related to mammalian CCL19 homologues than mammalian CCL21 homologues. Duck CCL19 is more closely related to chicken CCL19 than to chicken and duck CCL21. Chicken CCL19 and chicken CCL21 are approximately 35% identical to each other at the amino acid level. Also, the duck and chicken CCL19 are characterized by the presence of two cysteine residues in the leader peptide which is not observed in the mammalian CCL19 and CCL21 homologues. The chromosomal locations of the genes encoding CCL19 and CCL21 have been mapped in mice, humans and chickens (Nagira et al. 1997; Yoshida et al. 1997; Maho et al. 1999; Nakano and Gunn 2001; Wang et al. 2005). Both genes are adjacent to each other on mouse chromosome 4 in a region of conserved synteny to human chromosome 9p13 and chicken chromosome Z (Nagira et al. 1997; Yoshida et al. 1997; Maho et al. 1999; Nakano and Gunn 2001; Wang et al. 2005). These observations are consistent with the possibility that CCL19 and CCL21 arose from a common ancestral gene as a result of gene duplication during evolution.

The tissue distribution pattern of duck CCL19 was determined by northern blot analysis. Duck CCL19 has a very restricted expression profile in tissues. A

strong hybridization signal was detected only in spleen and a signal with a relatively weaker intensity was detected in the lung. In mice and humans, CCL19 is predominantly expressed in hematopoetic tissues (Yoshida et al. 1997; Ngo et al. 1998). Among the various tissues tested, the tissue distribution pattern of duck CCL19 was consistent with that of mammalian CCL19 homologues.

The copy number of genes encoding CCL19 in the duck genome was determined by Southern blotting analysis. In each digest, there were two hybridizing restriction fragments of different sizes for each of the enzymes. None of the enzymes used to digest the genomic DNA had cut sites in the probe. Studies in mice, humans and chickens have shown that CCL19 and CCL21 genes colocalize and are closely linked to each other (Nagira et al. 1997; Maho et al. 1999; Nakano and Gunn 2001; Wang et al. 2005). The genes have probably diverged during evolution from a common ancestral gene as a result of gene duplication. The nucleotide identity of the region used as a probe from duck CCL19 was compared to the duck CCL21 sequence. There was 36% nucleotide identity to the duck CCL21. It is not possible that the CCL19 probe is hybridizing to CCL21. Based on our data, it is most likely that there are two copies of CCL19 in the genome of ducks.

The biological function of recombinant CCL19 *in vitro* was assessed by chemotaxis assays using a transwell system. Recombinant CCL19 attracted freshly isolated duck peripheral blood mononuclear cells (PBMCs) in a dose dependent manner with maximum recruitment at a concentration of 10μg/ml. A significant response of duck PBMCs towards recombinant CCL19 in the presence

of a gradient confirmed that the migration was chemotactic and not random chemokinetic movement. There was significant migration of duck PBMCs to recombinant duck β_2 microglobulin subcloned into the same vector as recombinant CCL19 and expressed in *E. coli*. However, the response was abrogated by treatment of the β_2 microglobulin sample with Polymixin B sulfate, that binds to bacterial LPS in solution. Treatment of CCL19 sample with Polymixin B sulfate did not significantly alter chemotaxis. This implied that the chemotactic movement of duck PBMCs was not due to contaminating bacterial components from *E. coli*.

The variation in the number of responding cells to same concentrations of the chemokine in individual chemotaxis assays suggests that recombinant CCL19 was not completely refolded resulting in a partially bioactive form. However, every purified CCL19 preparation used in the chemotaxis assays was quantified to maintain consistency in concentration. The functional receptor for the CCL19 ligand is CCR7 (Yoshida et al. 1997). In humans, recombinant Flag-tagged CCL19 recruits CCR7 transfected cells *in vitro* at an optimal concentration of 300ng/ml (Yoshida et al. 1997). Cells from 5 different high power fields [400X magnification] were counted. The number of migrating cells in response to human CCL19 increased approximately six fold as the concentration was increased from 10 ng/ml to 300 ng/ml (Yoshida et al. 1997). In mice, a significant purified population of spleen T lymphocytes and a relatively lesser population of B lymphocytes migrated effectively towards recombinant CCL19 (Ngo et al. 1998). The percentage of input cells migrating towards mouse CCL19 was taken into

account. About 20% of input CD4 positive T cells migrated effectively towards 10ng/ml of recombinant CCL19, while approximately 40% of CD8 positive T cells exhibited significant migration at a concentration of 100ng/ml (Ngo et al. 1998). Recombinant duck CCL19 is required at a concentration of 1μg/ml to stimulate efficient chemotaxis. When the concentration of duck CCL19 was increased 10 times from 500ng/ml to 5 μg/ml, there was approximately a 2.5 fold increase in the number of duck PBMCs migrating in response to the chemokine. There are several possible reasons for this. Recombinant duck CCL19 expressed in *E. coli* was purified from inclusion bodies under denaturing conditions in 8M urea. The urea was removed by stepwise dialysis to a final concentration of 1M urea. Dialyzing recombinant CCL19 below 1M urea resulted in precipitation. Addition of a nonionic detergent n-octyl-β-D-glucopyranoside to the dialysis buffer to prevent aggregation below 1M urea was not successful.

The correct formation of disulphide bridges between the cysteines is critical for the biological activity of chemokines (Clark-Lewis et al. 1994; Clark-Lewis et al. 1995; Rajarathnam et al. 2001). The role of cysteines in the biological activity of chemokines has been well illustrated in IL-8 (Clark-Lewis et al. 1994). To assess the importance of individual disulphide bridges, the cysteine residues in IL-8 were substituted with α-aminobutyric acid [Aba]. These substituted forms lacked considerable activity and displayed marginal capability to interact with the cognate receptor as determined by ligand binding assays (Moser et al. 1991). Hence, it is speculated that the correct formation of disulphide bridges is necessary to provide the right framework and conformation for the receptor

binding motifs in the N-terminus and consequently important for biological activity of chemokines (Baysal and Atilgan 2001; Ott et al. 2004; Ott et al. 2004). CCL19 has four cysteine residues in the mature protein. The conformational restrictions imposed by the disulphides made it difficult to completely refold recombinant CCL19. The inability to refold recombinant CCL19 completely to its native conformation probably resulted in the requirement of higher optimal concentrations to induce chemotaxis. However, a consistently significant migration of PBMCs to recombinant duck CCL19 in comparison to the controls suggests that it is indeed biologically active.

We eliminated the possibility that the migration of duck PBMCs to our recombinant CCL19 was due to bacterial LPS contamination. Chemotaxis assays were performed with recombinant CCL19 and recombinant β_2 microglobulin with or without using Polymyxin B sulphate (Sigma) at a concentration of 10 micrograms/ml. Polymyxin B sulphate binds to bacterial LPS in solution. A significant response of duck PBMCs to endotoxin treated versus untreated recombinant CCL19 preparations confirmed that migration is not due to contaminating LPS from *E. coli*.

4.1.1 Expression of recombinant CCL19 in a eukaryotic system

The prokaryotic system enables expression of recombinant proteins in substantial amounts. However, a significant fraction of the protein in *E.coli* is recovered from inclusion bodies which are aggregates of malfolded polypeptides present in the insoluble fraction of the cell (Lu et al. 2001). The recombinant

protein is recovered under denaturing conditions and has to be refolded to its native conformation for biological activity. CCL19 has four cysteine residues in the mature protein. The correct formation of disulphide bridges between the cysteines is crucial for the biological activity of CCL19. The inability to refold recombinant CCL19 completely to its native conformation probably resulted in the requirement of higher optimal concentrations to induce chemotaxis.

Expressing recombinant CCL19 in a eukaryotic system is a potential alternative. Attempts to express recombinant human CCL19 in the bacculovirus expression system was unsuccessful (Yoshida et al. 1997). In mice, recombinant CCL19 has been expressed successfully using this approach. Briefly, the region encompassing the mature protein was expressed as an N terminal flag tagged CCL19 in HEK 293 cells. The cells were then transfected with pCEP-N-FLAG-CCL19 using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells which were resistant to puromycin were then expanded. Serum free supernatants were collected and recombinant N terminal flag tagged recombinant CCL19 was purified by affinity column chromatography using ANTI-FLAG M2-Agarose-sepharose (SIGMA) according to the manufacturer's instructions (Marsland et al. 2005).

In a most recent study in ducks, monoclonal antibodies have been developed to detect monocytes, T and B lymphocytes (Kothlow et al. 2005). These antibodies could be used to identify the phenotype of specific leukocyte subsets migrating in response to recombinant CCL19.

4.1.2 Characterization of duck CCR7, the receptor for duck CCL19

Generation of effective adaptive immune responses requires efficient orchestration of immune cells through the secondary lymphoid organs. Leukocyte recruitment to peripheral sites and to the lymph node is governed by the differential expression of chemokine receptors (Moser et al. 2004). In this regard, CCR7 plays an important role in guiding naïve lymphocytes and DCs to the secondary lymphoid tissues (Dieu et al. 1998; Sallusto et al. 1998; Sozzani et al. 1998; Yanagihara et al. 1998). CCR7 is the characteristic G protein coupled receptor for CCL19 and is expressed on the surface of naive T and B lymphocytes (Yoshida et al. 1997; Yanagihara et al. 1998; Yoshida et al. 1998). The mobilization of DCs from the periphery to the lymph nodes [LN] is regulated by CCR7. CCR7 expression is induced along with the maturation of human DCs while the expression of other chemokine receptors is down regulated (Dieu et al. 1998; Sallusto et al. 1998; Sozzani et al. 1998; Yanagihara et al. 1998). In mice, CCR7 is also selectively expressed by bone marrow derived DCs and epidermal LCs. In a recent study in mice, it was demonstrated that CCR7 is an essential mediator regulating the migration of skin (epidermal and dermal) DCs into the lymphatic vessels under inflammatory as well as steady state conditions (Ohl et al. 2004). Experimental evidence suggests that CCR7 deficient mice revealed a marked defect in migration of DCs to the LN (Forster et al. 1999; Ohl et al. 2004). In a separate study using mice as a model of asthma, the role of CCR7 in the exit of T cells from the periphery into the afferent lymphatics was established. In this

study, *in vivo* labeling of airway cells by carboxyfluoroscein succinimidyl ester [CFSE] demonstrated that peripheral T cells re-entered into the draining LN by migrating from the airways into the afferent lymphatic vessels. A significant population of these migrating T cells were CCR7 positive. The migration of effector T cells from the periphery was shown by the generation of transgenic mice that constitutively expressed CCR7 in T cells. The CCR7 positive T cells continued migrating into the afferent lymph and accumulated in the draining LN. To further substantiate this observation, competitive migration experiments were set up wherein both CCR7 positive and CCR7 negative T cells were directed into the lung airways. It was observed that only CCR7 positive T cells migrated from the lung and entered into the afferent lymphatic vessels (Bromley et al. 2005). Thus CCR7 expression is essential for DCs and T cells to migrate to the lymph node, it acts as one of the "gatekeepers" in modulating immune responses (Bromley et al. 2005; Lira 2005).

We have cloned and sequenced the duck homologue of CCR7 in our laboratory. The full length sequence of CCR7 cDNA was obtained by degenerate oligonucleotide RT-PCR and specific 5' and 3' RACE PCR. Sequence analysis reveals that there is high degree of sequence homology between duck CCR7 and other known CCR7 sequences in chicken, human, rhesus monkey, chimpanzee, pig, mouse and rat. The deduced amino acid sequence for duck CCR7 was compared to the schematic structure of human CCR7. Seven hydrophobic TMs, four ECs and four ICs including the extracellular amino-terminus and intracellular carboxy-terminus were identified in the duck CCR7. An analysis of the amino

acid alignment of various CCR7 sequences indicated conservation throughout most of the protein. In particular, comparison between duck CCR7 and human CCR7 at the amino acid level revealed identity in the amino acid residues of the extracellular domain [EC4] known to be important for CCR7 function. Northern blotting analysis indicated significant expression of duck CCR7 in the spleen and lung, consistent with the expression pattern of our duck CCL19 [Ross and Brusnyk, unpublished data].

To extend our findings about duck CCL19, we could further study CCR7-CCL19 receptor-ligand interactions. In humans, there has been a similar attempt to demonstrate affinity between the ligand CCL19 and its cognate receptor CCR7 (Ott et al. 2004 b). Briefly, chemotaxis assays were set up in which Jurkat T leukaemia cell line stably transfected with CCR7 by electroporation migrated towards different concentrations of CCL19. The cells were washed twice with PBS and resuspended in Dulbecco's modified Eagle Medium (Cellgro, Fisher Scientific) supplemented 2 mM L-glutamine, 10mM HEPES and 1mM sodium pyruvate and used in the chemotaxis assays to determine the migratory response to CCL19 (Ott et al. 2004 b).

4.1.3 Therapeutic potential of CCL19 – scope in DC based vaccines

Currently, the use of DC based vaccines for treatment of various infectious diseases has become an area of great interest because of its ability to induce both cellular and humoral immune responses. Activated DCs successfully prime T cells in the context of specific antigens [Ags] which is key to breaking tolerance

(Condon et al. 1996; Casares et al. 1997; Porgador et al. 1998; Akbari et al. 1999). The resting DCs are activated by adjuvants, inflammatory cytokines, chemokines, bacterial products, resulting in the upregulation of MHC-II molecules and co-stimulatory molecules (Sallusto and Lanzavecchia 1994; Gao et al. 2005; Okada et al. 2005). Therefore, manipulating the immune regulatory capacities of APCs using chemokines as immune modulators has immense scope in vaccine design and testing of antiviral therapies (Pinto et al. 2003; Frauenschuh et al. 2004).

CCL19 functions as a leukocyte chemoattractant recruiting professional antigen presenting cells [APCs] and T lymphocytes and to the secondary lymphoid tissues (Yoshida et al. 1997; Ngo et al. 1998; Yoshida et al. 1998; Kellermann et al. 1999; Iwasaki and Kelsall 2000; Baekkevold et al. 2001). CCL19 directs the migration of mature DCs to the distinct T cell zones in the lymphoid organs thereby establishing a functional microenvironment to prime näive T cells (Kellermann et al. 1999; Kaiser et al. 2005). CCR7, the functional receptor for CCL19 is upregulated on activated DCs and promotes their migration to the lymph node in response to CCL19 (Yanagihara et al. 1998; Marsland et al. 2005). CCR7 acts as a molecular gatekeeper guiding DC and T cell migrations from the periphery to the LN where it also promotes T cell priming in the context of specific Ags (Bromley et al. 2005; Debes et al. 2005; Lira 2005).

CCL19 has also been identified as a potent inducer of DC maturation triggering the upregulation of co-stimulatory molecules and involved in the release of pro-inflammatory cytokines such as IL-12 and TNF- α. The critical role of CCL19 and/or CCL21 as initiators of DC maturation is established by the

observation that DCs in *plt* mice fail to mature completely after peripheral stimulation with TLR ligands and reach the LNs in a semi-mature state (Marsland et al. 2005). Thus CCL19 and CCL21 play pivotal roles in driving the maturation of PAMP-licensed DCs. These CCL19 and CCL21 activated DCs effectively differentiate thereby triggering the secretion of pro-inflammatory cytokines that determine the direction of subsequent T cell responses. Specifically, these cytokines are potent in inducing cytotoxic T cell and Th-1 responses (Marsland et al. 2005). This pro-inflammatory role of CCL19 has implication in initiating effective, sustained cell mediated responses against viruses.

It has also been demonstrated that due to CCL19 secretion, activated DCs are able to induce a polarized and motile state in naïve T cells. In a recent study, video microscopy imaging system has been used to capture some of the early interactions between mature DCs and naïve T cells. Human naïve T cells were incubated along with autologous monocyte derived DCs. The response of naïve T cells to immature and mature DCs was observed and compared using a video microscopy imaging system. It was found that about 60% of naïve T cells became polarized and motile in the presence of mature, activated DCs. This resulted in an effective scanning of DC surfaces by T cells, thus enhancing the chances of MHC-peptide interactions. Neutralizing antibodies to CCL19 and CCR7 resulted in approximately 92% reduction in the polarization of T cell responses. These findings conclusively support the theory that CCL19 facilitates interactions between naïve T cells and cognate Ag bearing APCs (Kaiser et al. 2005).

These attributes of CCR7 and CCL19 makes them potential candidates for the development of Ag specific DC based vaccines. In this regard, the CCR7 ligands have demonstrated potential in orchestrating an effective antiviral immune response against HSV in mice. In these studies, plasmid DNA encoding CCL19 and CCL2 were codelivered intranasally with plasmid DNA encoding herpes simplex virus (HSV) gB (HSV-gB) in a prime-and-boost vaccination strategy. This vaccination strategy enhanced serum and vaginal IgG and IgA levels, as well as the numbers of HSV-$gB_{498-505}$ peptide-specific gamma interferon-producing $CD8^+$ T cells. Also, a significant number of cytotoxic T lymphocytes were observed when CCL19 and CCL21 was applied at both prime and boost in comparison to the absence of CCL19 and CCL21. These studies demonstrated that CCL19 and CCL21 expressed ectopically may serve as molecular adjuvants to boost the immune response to a codelivered antigen in mucosal surfaces (Eo et al. 2001; Toka et al. 2003).

Experimental evidence suggests that immunizing transgenic mice with hepatitis B surface antigens [HBsAg] by cytokine activated DCs can break tolerance and trigger an HBV specific CTL response (Shimizu et al. 1998; Engler et al. 2001). Cytokine activated, bone marrow derived DCs and freshly isolated splenic DCs from transgenic and non-transgenic mice were compared, in their ability to process and present the HBsAg. This was done by measuring the susceptibility of HBsAg pulsed DCs to cytolysis. Transgenic mice infused with *ex-vivo* activated DCs produce normal numbers of HBsAg specific CTLs. The *ex-vivo* activated DCs deliver efficient co-stimulatory signals to the anergic,

transgenic CTLs which enable them to mount a sustained, HBsAg specific CTL response (Shimizu et al. 1998).

In a separate study, adoptive transfer of DCs pulsed with human papilloma virus peptide [HPV], induced HPV specific CTL responses and protected mice from HPV induced tumors (Ludewig 2003). This indicates the involvement of DCs in the activation of anti-HPV T cells *in vivo*. Most DC based vaccine strategies require that the DCs be customized *in vitro*, which involves isolation of DCs and manipulation of their maturational states *in vitro*. Upon interaction with antigens, the DCs migrate to the draining lymph nodes to meet the T cells. The activated DCs secrete chemokines to which appropriate receptor bearing cells respond (Ngo et al. 1998). This is important to prime naïve T cells and initiate an antigen specific immune response.

Our interest is in using the duck model of hepatitis B for designing antiviral strategies. Identification of specific markers in duck immune cells is essential for the development of assays to characterize cell mediated immune responses. However, there are limitations in studying host immune responses to DHBV as sufficient immunological reagents are not available for the domestic duck *Anas platyrhynchos*. Functional characterization of novel chemokines and cytokines in ducks would enable effective use of the DHBV model.

Chemokines have been extensively studied in mammals. Among birds, there are few reports on chicken chemokines (Hughes et al. 2001; Kaiser et al. 2005) (Sick et al. 2000; Wang et al. 2005), but very little is known about duck chemokines. Only three members of the CC chemokine family CCL4, CCL5 and

MCP-1 have been identified, but not functionally characterized in ducks (Sreekumar et al. 2005). Characterization of duck CCR7 is a critical preliminary step towards obtaining specific markers for duck immune cells. This would be helpful in characterizing the cell mediated immune responses involving DC based vaccines. In this regard, this study is the first attempt in the functional characterization of duck CCL19. These findings can be extended towards the development of duck CCL19 as a molecular adjuvant to initiate an effective DHBV antigen specific immune response in ducks.

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